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An in vitro and in silico study

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Author's address: School of Pharmacy, Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND

Supervisors: Professor Hannu Raunio, M.D., Ph.D.
School of Pharmacy, Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND

Professor Risto Juvonen, Ph.D.
School of Pharmacy, Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND

Professor Maija Lahtela-Kakkonen, Ph.D.
School of Pharmacy, Faculty of Health Sciences
University of Eastern Finland
KUOPIO
FINLAND

Reviewers: Senior Scientist Päivi Taavitsainen, Ph.D.
Orion Pharma
Research and Development, Drug Disposition and Pharmacometrics
TURKU
FINLAND

Docent Jukka Mäenpää, M.D., Ph.D.
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Research and Development, Patient Safety
GOTHENBURG
SWEDEN

Opponent: Docent Miia Turpeinen, M.D., Ph.D.
Department of Pharmacology and Toxicology
University of Oulu and Oulu University Hospital
OULU
FINLAND

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ABSTRACT

Alkaloids have enjoyed a long history and remarkable role not only in the past but even in current medicine. They have been characterized as the most useful and also the most dangerous natural products. Many alkaloids have been used in herbal form long before their discovery as chemical molecules – and still today they are active ingredients in many herbal preparations, including those used in traditional Chinese medicine (TCM).

The use of herbal preparations (herbal medicinal products and food supplements) is widespread and has increased dramatically since the early 1990s also in the Western countries. This has generated significant and increasing concern about harmful drug interactions when these products are administered concurrently with conventional medicines since they can sometimes produce life-threatening consequences.

Most xenobiotics are extensively metabolized in humans. The human body processes all xenobiotics similarly, no matter the origin of a compound (i.e., natural or man-made). The liver is the principal site of xenobiotic metabolism and the majority of crucial steps in xenobiotic metabolism are undertaken by the cytochrome P450 (CYP) enzyme superfamily. Inhibition of these enzymes is the most common cause of metabolism-based drug interactions.

In this *in vitro* study, many isoquinoline alkaloids (N=34–49) were found to evoke significant inhibition of the most important human drug-metabolizing CYP enzymes; the rank order of the most potently inhibited enzymes was as follows: CYP3A4 > CYP2D6 > CYP2C19 > CYP1A2 > CYP2C9 > CYP2B6 > CYP2C8 > CYP2A6. There was a time-dependent inhibition and mechanism-based inactivation of CYP2C19, potentially the most alarming mode of inhibition. The results of this study indicate that herbal preparations may possibly be involved in clinically significant metabolism-based drug interactions.

This study adds to the growing information that natural does not always mean safe and recommends that closer attention should be paid to co-administration of herbal preparations with other medications; it is important that patients should inform and discuss about the use of these products with pharmacists and physicians.

In addition, this study introduces new laboratory and computational tools to screen for time-dependent inhibitors among early phase compounds in drug discovery and development programs.

National Library of Medicine Classification: QU 120, QU 143, QV 37.5, QV 38, QV 628
Medical Subject Headings: Isoquinolines; Cytochrome P-450 Enzyme Inhibitors; Metabolism/drug effects; Herb-Drug Interactions; High-Throughput Screening Assays; Quantitative Structure-Activity Relationship

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TIIVISTELMÄ

Alkaloideilla on merkittävä rooli paitsi muinaisessa myös nykyisessä lääketieteessä. Alkaloideja voidaan luonnehtia hyödyllisimmiksi ja vaarallisimmiksi luonnontuotteiksi; ne ovat olleet ensimmäisiä lääkkeitä, ensimmäisiä myrkkyjä ja ensimmäisiä torjunta-aineita. Monia alkaloideja on käytetty kasvimuodossa pitkään ennen niiden tunnistamista kemiallisina yhdisteinä – ja nykyäänkin ne ovat aktiivisia ainesosia monissa kasviperäisissä valmisteissa, mukaan lukien perinteisen kiinalaisen lääketieteen (TCM) valmisteet.

Kasviperäisten valmisteiden (kasvirohdosvalmisteet ja ravintolisät) käyttö on maailmanlaajuista ja lisääntynyt dramaattisesti varhain 1990-luvulta myös länsimaissa. Tämä on aiheuttanut merkittävän huolen haitallisista lääkeaineyhteisvaikutuksista yhteiskäytössä perinteisten lääkkeiden kanssa, koska yhteiskäytöllä voi olla myös hengenvaarallisia seurauksia.

Ihmiselimistö käsittelee tehokkaasti vierasaineita niiden poistamiseksi elimistöstä ja samalla tavalla riippumatta yhdisteen lähteestä (luonnontuote *vs.* synteettinen). Tämän biokemiallisen prosessoinnin eli vierasainemetabolian pääpaikka on maksa ja valtaosa kriittisistä vaiheista vierasainemetaboliassa on kytköksissä CYP-entsyymeihin. CYP-entsyymien toiminnan estäminen on kaikkein yleisin metabolia-perusteisten lääkeaineyhteisvaikutusten syy.

Tässä kokeellisessa (*in vitro*) tutkimuksessa monet isokinoliinialkaloidit (N=34–49) aiheuttivat merkittävää ihmisen vierasainemetaboliaan osallistuvien CYP-entsyymien toiminnan estymistä eli inhibitiota. Järjestys alkaen kaikkein voimakkaimmin inhiboiduista CYP-entsyymeistä oli seuraava: 3A4 > 2D6 > 2C19 > 1A2 > 2C9 > 2B6 > 2C8 > 2A6. Tarkemmissa tutkimuksissa havaittiin CYP2C19-entsyymin aika-riippuvaista inhibitiota ja katalyysi-riippuvaista inaktivaatiota, mitkä ovat nykyään kaikkein hälyttävimmät inhibition muodot. Nämä tutkimustulokset osoittavat, että kasviperäiset valmisteet voivat osallistua kliinisesti merkittäviin metabolia-välitteisiin lääkeaineyhteisvaikutuksiin.

Tämä tutkimus lisää tietoutta siitä, että luonnollinen ei aina tarkoita samaa kuin turvallinen. Tämä tutkimus kannustaa siten kiinnittämään tarkempaa huomiota kasviperäisten valmisteiden käyttöön yhdessä muiden lääkkeiden kanssa. On tärkeää, että potilaat kertovat ja keskustelevat näiden tuotteiden käytöstä farmaseuttien, proviisorien ja lääkäreiden kanssa.

Tämän lisäksi tämä tutkimus esittelee uusia laboratoriomenetelmiä ja tietokoneavusteisia työkaluja aika-riippuvaisten inhibiittorien seulomiseksi lääkekehityksen varhaisessa vaiheessa.

Luokitus: QU 120, QU 143, QV 37.5, QV 38, QV 628
Yleinen suomalainen asiasanasto: alkaloidit; sytokromit; entsyymit; inhibiittorit; yhteisvaikutukset; lääkekasvit; rohdosvalmisteet

"This is what the LORD says: –Stand at the crossroads and look; ask for the ancient paths, ask where the good way is, and walk in it, and you will find rest for your souls". –Jeremiah 6:16

"Näin sanoo Herra: –Pysähtykää ja katsokaa, minne olette menossa, ottakaa oppia menneistä ajoista! Valitkaa oikea tie ja kulkekaa sitä, niin löydätte rauhan." –Jeremia 6:16

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All the glory to the God.

Kuopio, November 2017
–in the Jubilee Year of Suomi Finland 100 and the Reformation 500 years–

Kaisa Salminen

List of the original publications

This dissertation is based on the following original publications:

I Salminen KA, Meyer A, Jerabkova L, Korhonen LE, Rahnasto M, Juvonen RO, Imming P and Raunio H. Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids. *Phytomedicine 18(6): 533-538, 2011.*

II Salminen KA, Leppänen J, Venäläinen JI, Pasanen M, Auriola S, Juvonen RO and Raunio H. Simple, direct, and informative method for the assessment of CYP2C19 enzyme inactivation kinetics. *Drug Metabolism and Disposition 39(3): 412-418, 2011.*

III Salminen KA, Meyer A, Imming P and Raunio H. CYP2C19 progress curve analysis and mechanism-based inactivation by three methylenedioxyphenyl compounds. *Drug Metabolism and Disposition 39(12): 2283-2289, 2011.*

IV Salminen KA, Rahnasto-Rilla M, Väänänen R, Imming P, Meyer A, Horling A, Poso A, Laitinen T, Raunio H and Lahtela-Kakkonen M. Time-dependent inhibition of CYP2C19 by isoquinoline alkaloids: in vitro and in silico analysis. *Drug Metabolism and Disposition 43(12): 1891-1904, 2015.*

Contents

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion and toxicity
ACN	Acetonitrile
ANF	α-Naphthoflavone
AUC	Area under the plasma concentration-time curve
BIA	Benzylisoquinoline alkaloid
CAM	Complementary and alternative medicine
cDNA	Complementary deoxyribonucleic acid
CLint	Intrinsic clearance
Cmax	Maximum plasma concentration
CoMFA	Comparative molecular field analysis
CoMSIA	Comparative Molecular Similarity Indices Analysis
CPIC	Clinical Pharmacogenetics Implementation Consortium
CPR	NADPH-cytochrome P450 reductase
CYP	Cytochrome P450
3D-QSAR	Three-dimensional quantitative structure activity relationship
DBF	Dibenzylfluorescein
DMSO	Dimethyl sulphoxide
EMA	European Medicines Agency
EM	Extensive metabolizers
FASS	Farmaseutiska Specialiteten i Sverige
FDA	The US Food and Drug Administration
GAP	Good agricultural practice
GMP	Good manufacturing practice
HLM	Human liver microsomes
HMP	Herbal medicinal product
HTS	High-throughput screening
IARC	International Agency for Research on Cancer
IC_{50}	Concentration of a compound causing to 50% inhibition
IM	Intermediate metabolizers
k_{deg}	Biological turnover rate of an enzyme
K_i	Inhibition constant
K_I	Inactivator concentration when the rate of inactivation reaches half the maximum
k_{inact}	Maximum inactivation rate
K_m	Michaelis-Menten constant for a substrate (concentration at which half-maximal velocity is achieved)

k_{obs}	Pseudo first-order rate constant of (slow-binding) inhibition
LC-MS	Liquid chromatography-mass spectrometry
LOO	Leave-one-out
MAO	Monoamine oxidase
MD	Molecular dynamics
MIC	Metabolite-intermediate complex
MBI	Mechanism-based inhibition
MDP	Methylenedioxyphenyl
NADPH	Nicotinamide adenine dinucleotide phosphate
NCE	New chemical entity
NP	Natural product
PASS	Post-authorization safety study
PDB	Protein Data Bank
P-gp	P-glycoprotein
PhRMA	Pharmaceutical Research and Manufacturers of America
PLS	Partial least square
PM	Poor metabolizers
PPI	Proton pump inhibitor
QM	Quantum mechanics
rhCYP	Recombinant human CYP
SNP	Single nucleotide polymorphism
SPE	Solid-phase extraction
TCM	Traditional Chinese medicine
TDI	Time-dependent inhibition
THMP	Traditional herbal medicinal product
TIA	Transient ischemic attack
TM	Traditional medicine
UM	Ultrarapid metabolizers
V_{max}	Maximal reaction velocity

1 Introduction

Xenobiotic is a general term describing any chemical which can interact with an organism and that does not occur in the normal metabolic pathways of that organism. Humans are constantly exposed to different kinds of xenobiotics from both natural and man-made sources for example, through their diet, the environment, and by contact with different kinds of products that contain these compounds, including drugs, natural products, food supplements, cosmetics, cleaning products, environmental pollutants, herbicides and pesticides, etc.

There is one group of xenobiotics that are used widely and increasingly: the herbal preparations (natural products, herbal medicinal products and food supplements). They have gained global popularity for promoting healthcare as well as preventing diseases – despite incomplete information concerning their active constituents, mechanisms of action, efficacy and safety. Herbal preparations have not been acknowledged earlier as medicines, which is probably one of the reasons why their efficacy and adverse effects including drug interactions are often underestimated. This may also have contributed to the false assumption that natural means safe. However, in the 1970s, drugs originated from plants (the digitalis glycoside digoxin and the alkaloid quinidine) were among the first reported to be involved in drug interactions. Since 2005, along with the changes in legislation in the EU (directive 2004/24/EY), herbal preparations have been divided into either drugs (medicinal products) or food products (botanicals).

Since ancient times, alkaloids have constituted an important group of natural products with a wide spectrum of biological activities varying from their abilities to elicit a medicinal cure, to non-medical use such as their hedonistic properties or even as poisons. Berberine, noscapine, morphine, quinine, cocaine, codeine, nicotine, caffeine and theobromine are only a few important examples of such alkaloids. Alkaloid-containing plants have been used in human medicine for thousands of years due to their potent biological activities, today not only do they still represent an important source of active pharmaceuticals but they are also interesting lead compounds in drug discovery programs. Many alkaloids have been used in herbal form for a long time before the elucidation of their active chemical constituents: they are the potential active ingredients in many herbal preparations worldwide, including preparations of traditional Chinese medicine (TCM) and Indian Ayurveda. (Benzyl)isoquinoline alkaloids have a long history of investigation in different research fields as well as a significant role in the past and current medicine.

Xenobiotics are often lipophilic – characteristics that promote their passage through biological membranes and hinder their excretion from the body. Thus, most of them are extensively metabolized in humans, and the human body processes all of them similarly, no matter the origin of a compound (i.e., nature or man-made).

Liver is the principal site of xenobiotic metabolism; the majority of crucial steps within xenobiotic metabolism are the responsibility of the cytochrome P450 (CYP) enzyme superfamily. The metabolism of drugs and other xenobiotics into more hydrophilic metabolites is crucial for the elimination of these compounds from the body and

termination of their biological activity. However, occasionally the metabolites are pharmacologically more active or even toxic than the parent compound.

One of the factors that can change the response to drugs is the concurrent administration of other xenobiotics. This can occur by competing for the same metabolic route, or via inhibition or induction of the activity of the xenobiotic metabolizing enzymes. Inhibition of CYP enzymes is the most common cause of metabolism-based drug interactions and is of clinical importance for both therapeutic and toxicological reasons. Whenever two or more drugs or other xenobiotics are administered over similar or overlapping time periods, there is a possibility for drug interactions. In addition to adverse effects, even deaths, during clinical drug use, drug interactions have resulted in early termination of drug development, refusal to obtain approval, severe prescribing restrictions and withdrawal of drugs from the market.

Based on the results of this study – the inhibition potential of the principal drug-metabolizing CYP enzymes – isoquinoline alkaloids have the potential to cause clinically significant drug-drug and herb-drug interactions.

It is clear that possible harmful interactions between multi-ingredient natural products and drugs is a topic of paramount importance. In order to optimize the use of herbal preparations, further studies and new methods to explore their properties and actions in the human body are necessary.

History forms the basis of our profession today. Knowledge is the way to update the past to the present; the past can not only help us to understand the present but also point the way to the future. Thus, the following review of the literature provides a short summary of the fascinating history of medicines along with natural alkaloids, a review of traditional medicines and natural products/herbal medicinal products with current regulatory status as well as other possibilities and challenges, a review of CYP enzymes and drug-drug and herb-drug interactions as well as the current status of *in vitro* (laboratory) and *in silico* (computational) possibilities.

2 Review of the Literature

2.1 NATURE AS A SOURCE OF MEDICINES

2.1.1 Natural products (NPs)

The term "natural product(s)", abbreviated as "NP(s)" will be used throughout this thesis. However, there is no consensus about the definition or even the meaning of the term "natural product". NP refers to single compounds or ingredients and products with single (active) ingredient or multicomponent mixtures from natural sources used e.g. for non-nutritive health benefits, such as prevention or treatment of health conditions. Thus, NPs can encompass also some country-specific and regional terms such as "dietary supplements" or "food supplements" (ravintolisä in Finnish), "herbal medicinal products" (kasvirohdosvalmiste in Finnish), "natural health products", "herbal/botanical products", "traditional medicines", "alternative/complimentary medicines", etc., as well as conventional medicines (Roe et al., 2016).

Plants are Mother Nature's supreme organic chemists – as evidenced by their ability to synthesize sophisticated carbon compounds with carbon dioxide as their sole carbon source – and they are capable of producing a myriad of chemical compounds via their sophisticated metabolic processes (see e.g. Figure 2, page 11). Higher plants produce diverse classes of metabolites which have proved to be bioactive compounds. While these metabolites serve specific functions in the plants, many have surprising effects on the human body. The primary metabolites (e.g. amino acids) are mainly required for the growth and development of the plant whereas secondary metabolites (e.g. NPs, alkaloids, phenolics and terpenoids) play a major role in the adaptation of plants to their environment, for example combating herbivores, pests and pathogens. Secondary metabolites have been exploited by humans for many purposes. One major characteristic of the secondary compounds is that their synthesis is highly inducible and their production varies not only between species but even in different geographical growing locations of the same plant. The versatile secondary metabolism of plants has been a treasure-trove for NPs throughout human history. There are 250000 living plant species and they produce a larger diversity of bioactive compounds than any man-made chemical library. Few researchers doubt that nature and plants are superior sources of molecular diversity and novel molecular chemotypes, particularly in those areas where good synthetic leads do not exist (Raskin et al., 2002).

The medical use of NPs, compounds derived from natural sources (e.g. plants or micro-organisms), precedes documented human history. Due to their diverse biological activities and medicinal potential, nearly every civilization has accumulated experience and knowledge of their use. Indeed, medicinal plants have yielded numerous valuable agents in the treatment of important human ailments. It is worth noting that a remarkable number of modern drugs have been isolated from natural sources and many of them had been used for centuries in traditional medicine (Raskin et al., 2002; Ji et al., 2009). Ultimately some of these drugs are such a part of our modern medicine (e.g., atropine, quinine, morphine and reserpine, etc.) that we may tend to forget their historical roots.

2.1.2 History of medicines along with natural products

Early history. Ancient Greece is the cradle of modern European medicine. However, the oldest medical text originates from ancient Mesopotamia, circa 2600 BC. It describes approximately 1000 plants and plant-derived substances and many of those herbs and formulations are still used today. One of those is the juice of the poppy seed *Papaver somniferum* (opium poppy, oopiumiunikko in Finnish; source for morphine and other important alkaloids to this day) (Newman et al., 2000). The ancient Egyptian *Ebers Papyrus*, dating from circa 1550 BC, contains approximately 800 complex prescriptions and more than 700 natural agents, such as *Aloe vera* (aloe) and the oil of *Ricinus communis* (castor oil, risiiniöljy in Finnish). Hippocrates of Cos (circa 460–377 BC), the famous Greek physician, collected more than 400 natural agents and described their use in *Corpus Hippocraticum* (i.e., a collection of early Ancient Greek medical works). For example, he detailed how to use an extract from *Atropa belladonna* (belladonna; source of atropine and other alkaloids) as an anaesthetic. Roman physicians built on this extensive knowledge and added their own understandings and experience. The Greek physician Pedanius Dioscorides (circa 40–90 AD) compiled *De Materia Medica*, which described the medicinal properties of about 600 plant-derived medicines, there by setting the foundations of pharmacology in Europe. Another famous Greek pathologist-physician, Galen (129–200 AD), recorded 540 plant-derived medicines and demonstrated that herbal extracts contain not only beneficial components, but also harmful ingredients (Ji et al., 2009). However, despite their attempts at objectivity and attempting to rationally explain medical phenomena, they tended to resort to irrationality when they tried to explain effects that were unexplicable to them. For example, their effects were associated by Hippocrates and Galen with climatic conditions and elements such as earth, water, air and fire (Boulogne, 2001). However, even today, it is obvious that modern medicine still faces limits in what is available to observation, even though those limits are constantly being extended. Although science cannot determine absolute truths, it can demonstrate which hypothesis is wrong.

Undoubtedly, Galen is a giant in the history of medicine but he casts a long shadow; his medical theories dominated but also prevented almost all progress in European medicine for 1500 years (Hajar, 2012). Galen's legacy was challenged by Philippus Theophrastus Bombastus von Hohenheim (better known as Aureolus Paracelsus) (circa 1497–1541); he criticized the medical establishment and pioneered the use of chemicals – both organic and inorganic – in medicine, particularly metals such as antimony and mercury, but not forgetting folk medicine (such as opium tincture). His explorations and procedures mean that he is recognized as the father of modern chemistry (Dominiczak, 2011). The statement that "the dose makes the poison" comes from Paracelsus, even today, this principle represents the basis for the establishment of exposure limits for (industrial) compounds (Weisburger, 2000).

Botanical taxonomy, i.e. the forms of scientific names of plants we use today, was invented and devised by the Swedish botanist and physician Carl von Linne´ (Carolus Linnaeus) (1707-1778), first in *Species plantarum* (1753) and later in various editions of *Systema naturae* (1758). Linné's systematic approach – binomial naming system, Linnaean names – enabled scientists throughout the world to communicate and immediately identify a plant or an animal by its Latin name. By structuring the naming of the plants he also

provided a unique basis for later physicians to identify herbs and plants with important biomedical properties (Knapp et al., 2004; Rossner, 2006).

Many of the medicines available to physicians in the 18th-century were derived from local plants and it was a practitioner's duty to be aware of their benefits. Investigating folklore claims led to the discovery of several drugs (Cragg et al., 2014). The lessons that the physician and botanist William Withering (1741–1799) learned from his studies of *Digitalis purpureaa* (foxglove; sormustinkukka in Finnish) are still relevant today. He published a monograph describing the clinical effects of an extract of the foxglove plant as well as the indication and the toxicity of digitalis in his book which appeared in 1785. Nonetheless, even before Withering, foxglove had been used in folk medicine as a remedy for dropsy (vesipöhö in Finnish), in today's medical jargon – cardiac insufficiency (sydämen vajaatoiminta in Finnish) (Breckenridge, 2006; Weisse, 2010).

Historically, plants represent the origin of modern pharmacotherapy. However, without simultaneous developments in many branches of natural science, this would have not been possible. The first commercial pure NP (morphine by Merck in 1826) and the first semisynthetic drug based on a NP (aspirin by Bayer in 1899) started a new era in medicine. In this new era, drugs could be purified from plants and administered in precise doses, independent of the source or age of the plant material. Modern analytical and structural chemistry have provided the tools to purify various constituents as well as elucidating their structures. In turn, this has revealed insights into their actions in the human body. The discovery, development and mass production of chemically pure NPs and chemically synthesized drugs profoundly altered health care (Fowler, 2006; Cragg et al., 2014).

During the 1810s, researchers investigated a new group of substances with dramatic physiological effects: the alkaloids. Morphine played an important role also in Magendie's (1783–1855) and Orfila's (1787–1852) experimental research; these were some of the first scientifically conducted poisoning trials and also one of the first criminal uses of the recently-discovered alkaloids. Thanks to advances in analytical procedures, the alkaloids could be isolated and administered in small controlled doses, for example making possible not only the refined animal experiments as conducted by Francois Magendie (one of the instigators of experimental medicine) but also a new therapeutic approach which he described in his popular and influential Formulaire in 1821 (Bertomeu-Sanchez, 2012).

Recent history. Until the 1990s, plants were a major source for the discovery of novel pharmaceuticals, with many important drugs having been derived directly or indirectly from plants. During the 20th century, the strong historical association between plants and human health began to unravel and the emphasis gradually shifted from extracting plant compounds to producing the same compounds or their analogues synthetically. NPs were then regarded as templates to allow structural optimization intended to devise new drugs. These are referred to as new chemical entities (NCEs). Combinatorial chemistry and computational drug design has brought an end to the dominance of NPs in drug discovery. The expansion of synthetic medicinal chemistry in the 1990s almost replaced the discovery and identification of new metabolites from living organisms – at this time, the pharmaceutical industry focused mainly on synthetic compounds as a source of new drugs. Simultaneously, there was a declining trend in the number of new drugs reaching the market, and this decline reinvigorated scientific interest in drug discovery from natural sources. It has even been postulated that the transition from NPs to combinatorial

chemistry during the 1990s might have led to the current lack of new drug candidates (Desai and Chackalamannil, 2008; Ji et al., 2009).

The biosynthetic pathway diversity in plants provides a variety of bioactive compounds; these can be used directly as therapeutic agents, and also as starting materials for the synthesis of drugs or as models for novel pharmacologically active compounds. Still, today's drugs either directly derived and used without modification from plants account for about 6% of all drugs and cover a range of applications from analgesics (codeine, morphine) to anti-cancer agents (taxol) and contraceptives (diosgenin) (Fowler, 2006). While in many of these cases, alternative routes of chemical synthesis have been developed, none have provided a viable commercial alternative which would replace direct use from the plant. The plant kingdom still remains a treasure-trove of new molecules with therapeutic potential and invaluable source of inspiration as approximately only 6% of all 250000 plant species have been studied for biological activity and approximately 15% have been studied phytochemically (Verpoorte, 2000; Stevigny et al., 2005; Newman and Cragg, 2007; Newman and Cragg, 2012; Newman and Cragg, 2016). The current importance of NPs is reflected when it is considered that ~50% of all the new drugs approved for clinical use in Western countries and Japan during the thirty-three year period from 1981 to 2014 were either NPs or their derivatives (25%), botanical drugs (defined mixtures) (1%) or synthesized molecules based on NP mimics or pharmacophores (25%) (Newman and Cragg, 2016). Although the growth of synthetic medicinal chemistry in the 1990s meant that the proportion of new drugs based on NPs declined to ~50%, 13 NP derived drugs were approved between 2005 and 2007 in the United States. Five of them were the first members of new classes (Li and Vederas 2009). These were the peptides exenatide (Bydureon® and Byetta®) and ziconotide (Prialt®) and the small molecules, ixabepilone (Ixempra®), retapamulin (Altargo®) and trabectedin (Yondelis®) (Harvey, 2008). There have been a total of 19 approvals of NPs or NP-derived drugs between 2005 and 2010, indicating that in spite of the dismissal of NPs by big pharma, NPs still continue to provide a good source of new drugs today (Mishra and Tiwari, 2011).

Good recent evidence and an example of the huge potential, resurgence and interest towards natural sources and traditional medicines are the Nobel laureates (William C. Campbell, Satoshi Omura, and Youyou Tu) of the 2015 Nobel Prize in Physiology or Medicine for their discovery of the NPs – avermectins and artemisinin, respectively – therapies that revolutionized the treatment of devastating parasite diseases. The Chinese scientist, Youyou Tu, searched the ancient literature on herbal medicine in her research to develop a novel cure for malaria. The plant *Artemisia annua* proved to be an interesting candidate, and Tu developed a purification procedure. This led to the discovery of the active agent, artemisinin, i.e., a drug that represented a new class of antimalarial agent with unprecedented potency in the treatment of severe malaria (Efferth et al., 2015; Shen, 2015).

Another fascinating example is paclitaxel from *Taxus brevifolia* (Pacific or Western Yew, lännenmarjakuusi in Finnish), a member of the taxane class and one of the most efficacious chemotherapeutic agents used against many forms of cancer. It was discovered as part of a National Cancer Institute screening program in the search for plant extracts with antineoplastic activity, and it has revolutionized anticancer treatment (Wani and Horwitz, 2014; Barbuti and Chen, 2015).

Despite the current preoccupation with synthetic chemistry in drug development, the contribution of plants in the search for new drugs is still huge (Raskin et al., 2002).

Understanding the effects of NPs is a matter of great scientific, economic and medical interest. It is predictable that the identification of new metabolites from living organisms will be at the core of pharmaceutical discovery efforts also in the future (Li and Vederas, 2009).

2.1.3 Alkaloids – a treasure-trove of poisons and medicines

Scientists are recognizing the vital importance of alkaloids for biology, medicine and chemistry. Alkaloids constitute a totally unique fascinating world with a wide variety of versatile and biologically, pharmacologically, physiologically and chemically active compounds. Thus, it is only possible to provide a brief overview of this fascinating and multidisciplinary research field.

There are probably over 25000 alkaloids produced by higher plants; alkaloids are found in about 20% of plant species. Plants that contain more than 0.01% of alkaloids are called alkaloid plants. Alkaloid-containing plant species are distributed worldwide and throughout the temperate areas (Ziegler and Facchini, 2008; Funayama and Cordell, 2015).

The NPs classified as alkaloids are an integral part of our everyday lives, as medicines, dyestuffs, flavors, stimulants and toxic substances. Alkaloids are probably best known for their biological activity. As seen from the history above, alkaloids have always fascinated those with an inquiring mind, and still, nowadays, they are an object of considerable scientific and economic interest. Since ancient times, alkaloids were the first medicines, the first poisons, and the first protection agents; they can be characterized as the most useful and also the most dangerous natural products. Plants that contain alkaloids have been used in human medicine for thousands of years due to their potent biological activities, and they still represent an important source of active pharmaceuticals. For example, opium poppy still remains the only commercial source for the narcotic analgesics alkaloids morphine, codeine and semi-synthetic derivatives such as oxycodone and naltrexone (Ji et al., 2009; Beaudoin and Facchini, 2014). Morphine – a prototype of the most potent class of analgesics – represents the first modern medicine and it is still in routine therapeutic use (Zenk and Juenger, 2007; Funayama and Cordell, 2015).

It is obvious that without alkaloids, remarkable victories in the battles against malaria, leukaemia and cancer would not have occurred. For example, vinca alkaloids represent one of the oldest classes of antineoplastic agents used in humans with a wide spectrum of activity against both animal and human tumors (Budman, 1997). Most (~80%) of the drugs prescribed today for cancer treatment, are based on NPs or mimetics, many of which have been improved with combinatorial chemistry. Plant-derived agents are still among the most effective cancer chemotherapeutics currently available (Cragg et al., 2014). Undoubtedly, alkaloids will also play an important role in the future research and development and new applications will emerge for protecting human health and welfare (Zenk and Juenger, 2007; Funayama and Cordell, 2015).

Many alkaloids are still being dispensed in a herbal form; alkaloids are potential active ingredients in many NPs. For example, the alkaloid quinine was used in a herbal form by the native people of America many hundred years before it was recognized by scientists in the West. The possibility of using this herbal medicine to cure of malaria can be traced to

1638, when some aristocrats were cured of malaria after treatment with the plant extract (Aniszewski, 2015).

2.1.3.1 Definition

The definition of the term alkaloid is not simple, and has even changed significantly over the years, as more "alkaloids" have been structurally elucidated and the sources of alkaloids have broadened. Thus, a short exact and all-inclusive definition is not even possible nowadays without providing a long list of exceptions. This is understandable taking into account the great number of currently known alkaloids (approximately 25000), the biological and chemical nature of this group of compounds and their vital importance in the fields of biology, medicine and chemistry. The term "alkaloid" originates from the time of the discovery of these compounds (in the early 1800s), when alkaloids were discovered only from higher plants, and those compounds showed basic properties and strong biological activities. Thus, the name 'alkaloid' derives originally from the word alkaline (referring to "alkali like") and an alkaloid was originally defined as "the plant component which shows basic properties and strong biological effect". Alkaloids are no longer limited only to those NPs which are basic in character, and currently alkaloids have been obtained from an extremely broad range of natural sources, not just the plant kingdom. Although these compounds are often difficult to synthesize ex vivo, the progress in both theoretical and applied chemistry has led to the synthesis novel variations which do not exist in the nature, adding to the list of existing alkaloids. From the biological point of view, an alkaloid can be described as any biologically active and heterocyclic chemical compound that contains nitrogen and may have some pharmacological or toxicological activity and, in many cases, medicinal or ecological use (Aniszewski, 2015).

Difficulties with the definition of such a group of natural molecules as alkaloids also stem from the similarities between alkaloids and other secondary compounds. Alkaloids can exist in complexes with different moieties, such as peptides, proteins and sugars. Additionally, alkaloids were used for a long time before their discovery as chemical molecules with their own unique nomenclature. It is worth of noting that amino acids, peptides, nucleosides, amino sugars and antibiotics are not considered to be alkaloids (Aniszewski, 2015).

Natural alkaloids are all alkaloids existing and naturally synthesized in nature, currently known or still unknown. **Biomimic alkaloids** are natural alkaloids copied artificially by chemists; they are identical in structure to natural alkaloids. **Bionic alkaloids** are those biomimic molecules produced by chemists and bioengineers using natural models and high-level technology. Bionic alkaloids are analogues but they are not identical to natural alkaloids. **Synthetic alkaloids** are molecules fully modeled by chemists and bioengineers using high-level technology, planned models and artificial synthesis procedures. Synthetic alkaloids are not produced naturally by any living organism (Aniszewski, 2015).

2.1.3.2 Classification

Due to both biological and chemical nature, their long history and diverse roles and applications of alkaloids, several approaches to the classification of alkaloids are available, e.g. chemical, taxonomic, biological and biosynthetic nomenclatures. Alkaloids can be classified in several ways e.g. in terms of their 1) chemical structure, 2) biosynthetic pathway, 3) biological and ecological activity and 4) relation to chemical and technological innovations. The following paragraphs summarize the basic ideas behind the chemical, biosynthetic and taxonomic classifications.

Chemical structure. Alkaloids are often classified on the basis of their chemical structure, typically common heterocyclic nucleus, such as isoquinoline, indole, quinolone, quinazoline, pyrrolizidine and tropane alkaloids, etc. The backbone structures of the principal alkaloids are presented in Figure 1.

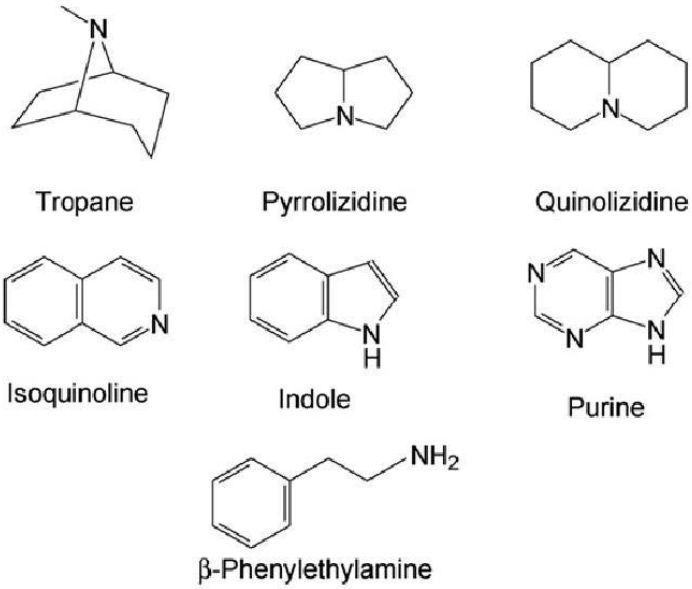


Figure 1. Backbone structures of the principal alkaloids (Gotti, 2011).

Biosynthetic pathway. Alkaloids can be generally classified on the basis of their biosynthetic origin or pathways according to their precursors and divided into three main types: **true alkaloids**, **protoalkaloids** and **pseudoalkaloids**. Both true and protoalkaloids are synthesized mainly from the aromatic amino acids, i.e phenylalanine, tyrosine (isoquinoline alkaloids), tryptophan (indole alkaloids), lysine (piperidine and quinolizidine alkaloids) and ornithine (pyrrolidine, pyrrolizidine and tropane alkaloids). The precursor amino acid does not determine whether the alkaloid is a true alkaloid or protoalkaloid. The main difference between the true alkaloids and protoalkaloids is that true alkaloids share a heterocyclic ring with nitrogen, and in protoalkaloids, the N atom derived from the amino acid is not a part of the heterocyclic bond. The basic carbon skeletons in pseudoalkaloids are not derived from amino acids. Pseudoalkaloids are associated with amino acid pathways so that they are derived from the precursors or postcursors of amino acids;

transamination reactions precede pseudoalkaloids. Pseudoalkaloids can also be synthesized from other compounds (nonaminoacid precursors), for example, acetate in the case of some piperidine alkaloids (coniine or pinidine). In addition, pseudoalkaloids can be phenylalanine-derived or steroidal or terpenoid alkaloids. Protoalkaloids form a minority of these three types of alkaloids. True alkaloids are typically highly reactive substances with biological activity, even at low concentrations. All three types of alkaloids (true alkaloids, protoalkaloids and pseudoalkaloids) can be as natural, biomimic, bionic and synthetic alkaloids.

Natural origin. One possible method to classify the alkaloids is to use their natural origin. It is possible to organize them, for example, based on a plant family, such as Amaryllidaceae, Solanaceae and Rutaceae alkaloids, or based on a genus, such the *Catharanthus* alkaloids. The following is one example of a taxonomic classification from order to a single plant species; Order: Ranunculale → Family: Papaveraceae → Subfamily: Papaveroideae → Genus: Papaver → and Plant species: *Papaver somniferum*. A large plant family may produce several groups of structurally diverse alkaloids. At the same time, one particular alkaloid can be produced by various plant families. Consequently, if one wishes an overall alkaloid classification, this cannot be considered as an effective approach.

Classification may use also the name of a **prototypical alkaloid** of the group such as aconitine-type or morphine-type alkaloids as well as aporphine, protoberberine, and benzophenanthiridine alkaloids. Frequently, this approach also follows a common biosynthetic or biogenetic origin. For example (*S*)-reticuline is the common biosynthetic precursor of the aporphine-, morphine-, protoberberine- and benzophenanthiridine-type alkaloids (Aniszewski, 2015).

Some of the above concepts are gathered into Table 1.

Table 1. Some general concepts related to alkaloids.

Concept	**Definition**
Natural alkaloid	All alkaloids existing and naturally synthesized in nature, currently known or still unknown
Biomimic alkaloid	Natural alkaloid copied artificially by chemists in the laboratory, but identical in structure to natural alkaloid
Bionic alkaloid	Biomimic molecule being novelized by the chemists and bioengineers using natural models and high-level technology
Synthetic alkaloid	Molecule fully modeled by chemists and bioengineers using high-level technology, planned models and artificial synthesis; not produced naturally by any living organism
True alkaloid	Synthesized from the aromatic amino acids; have a heterocyclic ring with nitrogen from the precursor
Protoalkaloid	Synthesized from the aromatic amino acids; N atom derived from an amino acid is not part of the heterocyclic bond
Pseudoalkaloid	Can be synthesized from other compounds (i.e., non-amino acid precursors)

2.1.3.3 Complexity of alkaloids

At present, the metabolic pathways of alkaloids are relatively well understood from the point of view of organic chemistry (Figure 2). But many questions remain relating to the biological nature of alkaloid synthesis; most plant alkaloids are derived through highly complicated biosynthetic pathways (Aniszewski, 2015). For example, the pathway to morphine comprises a total of 19 chemical steps which start from 2 molecules of L-tyrosine to the molecule of morphine that contains 5 asymmetric centers. For example, in 2007, two steps, the vinyl ether cleavage enzyme and the codeine demethylation steps in the poppy plant, were still unclear (Zenk and Juenger, 2007). It was only recently that Winzer et al. (2015) identified the final key enzyme in the poppy's biosynthetic pathway for morphine; the work revealed an unusual protein that contains both cytochrome P450 and oxidoreductase modules. *Papaver somniferum* is still the most economically viable source of the painkiller morphine. The identification of this enzyme and the final step may enable alternate routes for morphine biosynthesis that are less dependent on poppy cultivation.

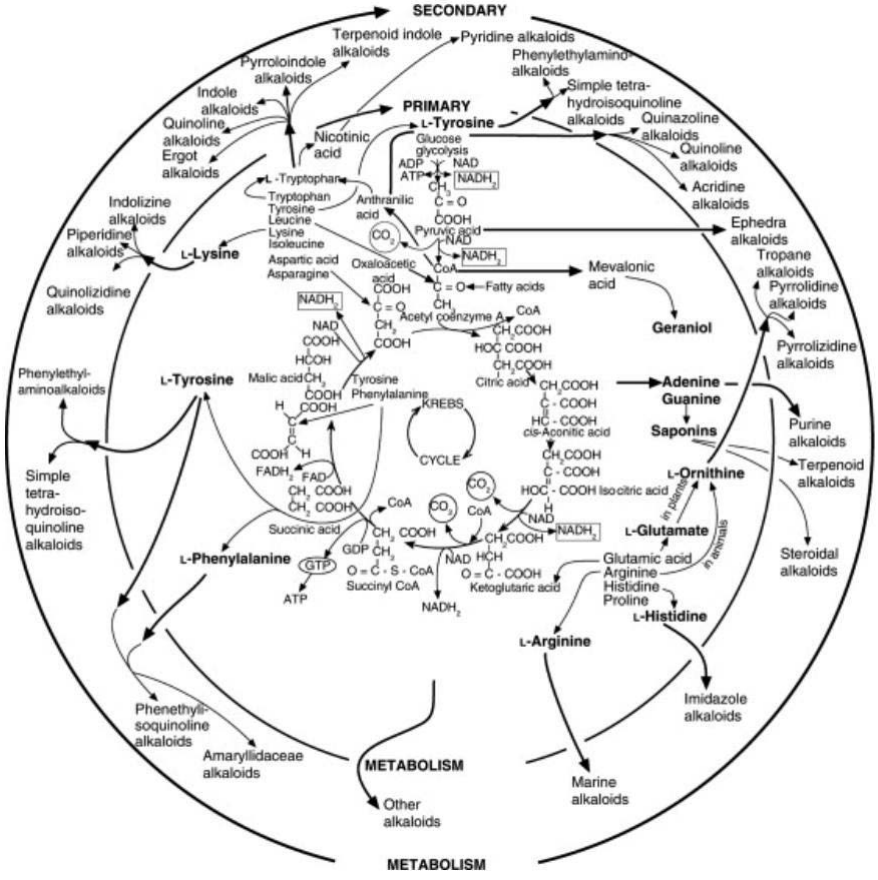


Figure 2. General scheme of alkaloid synthesis (Aniszewski, 2015).

2.1.3.4 Benzylisoquinoline alkaloids (BIAs)

Benzylisoquinoline alkaloids (BIAs) are a large and structurally diverse specialized group of plant metabolites of which approximately 2500 members have been identified (Ziegler et al., 2009); see for example a database for BIAs available on-line: http://crdd.osdd.net/raghava/biadb/index.html (Singla et al., 2010). BIAs are one of the most typical alkaloids derived from phenylalanine and tyrosine. Although the ecophysiological functions of most BIAs are unknown, they have a long history of investigation in different research fields and they play a significant role not only in the past but also in current medicine. They are among the most important NPs with extensive traditional clinical use stretching back thousands of years and this has been validated in modern evidence-based medicine studies. Many BIAs possess potent pharmacological effects and are in widespread clinical use, including most notably the opiates (narcotic analgesics) morphine, codeine and their semi-synthetic derivatives such as oxycodone and naltrexone, the cough suppressant noscapine, the vasodilator (muscle relaxant) papaverine, the antimicrobial agent sanguinarine and apomorphine (a derivative of morphine) used in Parkinson's disease (Hagel and Facchini, 2013). In addition, berberine has been one of the most widely studied alkaloids and is being used as a herbal medicinal product (Zou et al., 2017).

BIAs are found in many plant families and genera and probably in hundreds or thousands of species all over the world. For example, all plants of the Papaveraceae family are reported to be extensive alkaloid producers. Many BIAs of pharmaceutical importance possess one or more chiral centers, which preclude chemical synthesis as an economically practical alternative for commercial production (Hagel and Facchini, 2013). Figure 3 illustrates some BIA structural subgroups.

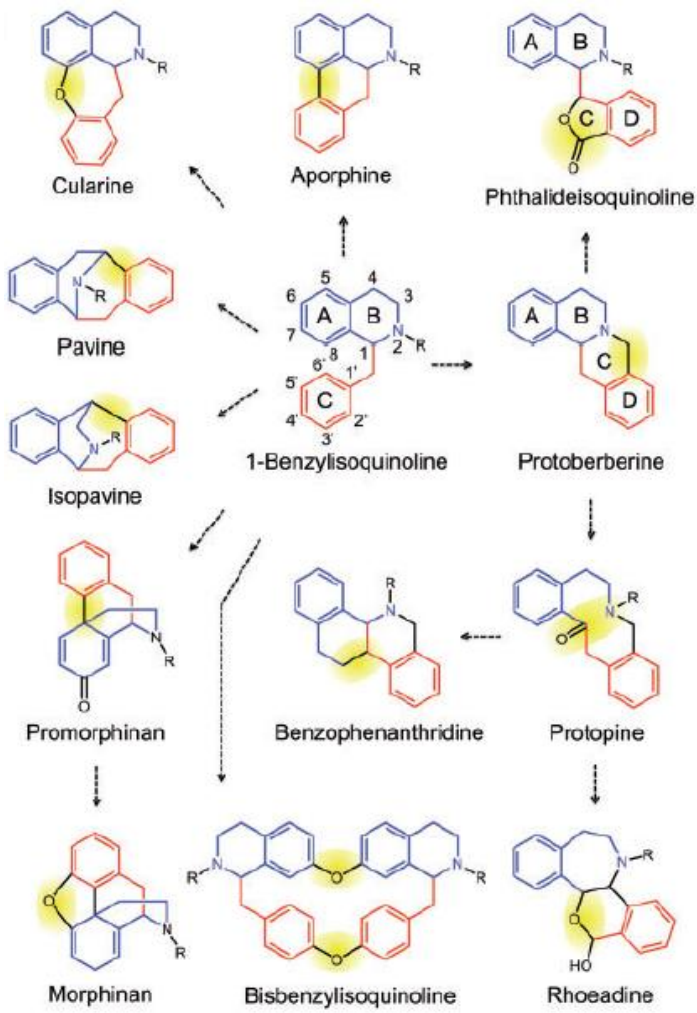


Figure 3. Benzylisoquinoline alkaloid structural subgroups derived from the basic benzylisoquinoline subunit. Blue and red designate the parts of each molecule originating from the tetrahydroisoquinoline moiety and the benzylic moiety, respectively. Yellow highlights express landmark C–C or C–O bonds formed in the benzylisoquinoline subunit and defining each structural subgroup (Hagel and Facchini, 2013).

2.1.4 Traditional medicines

Traditional Chinese medicine (TCM) (Kampo in Japan) and Indian Ayurveda are characterized by their extensive use of medicinal plants. The use of a systematic mixture of crude drugs forms the basis of many aspects of TCM. Herbal medicine as a part of TCM dates back as far as 2100 BC. Some 7000 different plant species and more than 13000 different herbal preparations are used in TCM. Preparations may vary extensively with regard to composition and dosage as well as processing, which may include, for example, stir-frying or soaking in vinegar or wine (Stickel et al., 2000; Stickel and Shouval, 2015). In addition, herbal products are used often as individualized remedies. Although the ancient Occidental (Western) and Oriental (Eastern) medicinal systems developed independently of each other, it is interesting that their respective practitioners often used the same NPs to treat similar diseases. For example, both *Shen Nong Ben Cao Jing* (i.e., ancient Chinese

medical literature classic) and *De Materia Medica* (by Greek physician Dioscorides) describe the use of an extract from Tussilago farfara (leskenlehti in Finnish) as an antitussive to suppress coughing. Hippocrates used an extract of Veratrum album (white hellebore; valkopärskäjuuri) as an emetic, whereas his Chinese colleagues used an extract of Veratrum nigrum (black hellebore; tummapärskäjuuri in Finnish) to treat the same ailment. The oil of Nepeta cataria (catnip; aitokissanminttu in Finnish) has been used as an antipyretic in Europe for a long time, and *Shen Nong Ben Cao Jing* notes the same use for another species of the family, Nepeta tenuifolia (Ji et al., 2009).

Currently, there are fundamental differences between Western medicine and traditional medicines as well as their remedies and medications, because these are based on different cultures. Traditional medicine is defined by WHO in the 'WHO Traditional Medicine Strategy 2014–2023' (released 2013 and available over the Internet: http://www.who.int/medicines/publications/traditional/trm_strategy14_23/en/) as follows: "Traditional medicine has a long history. It is the sum total of the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses." The terms complementary/alternative/non-conventional medicine are interchangeable with traditional medicine. Figure 4 illustrates certain members of the plant kingdom as a source of medicines (systems of medicines) and drugs with basic characteristics as well as their connections and differences.

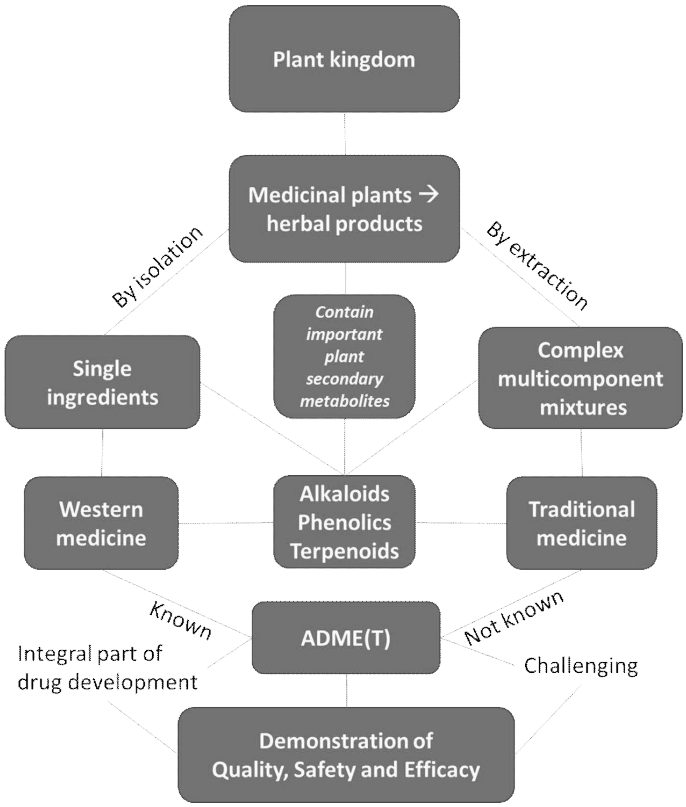


Figure 4. Schematic framework of some basic characteristics and main differences of systems of medicines and their drugs. ADMET: absorption, distribution, metabolism, excretion and toxicity.

WHO has estimated that approximately 80% of the populations in developing countries rely on traditional medicines, mainly from herbal sources, in their primary healthcare: due to cultural tradition or a lack of alternatives. Natural product use has multiplied in the general population during the last decades also in developed countries, including regions like North America and Europe where Western medicine is primarily practiced (Manohar et al., 2015). The rising popularity of oriental medicines in general has promoted also the use of TCM worldwide. Currently, billions of dollars are spent on botanicals and related products; an estimated total value of the world market for herbal products stands at around 83 billion US dollars and Europe accounts for over 50% of that total (WHO, 2004; De Vos, 2010; Pelkonen et al., 2014; Coutinho Moraes et al., 2015). Concomitantly with the continuous growth of (herbal) food supplement sales, there has been an exponential increase of their number on the market from around 4000 in 1994, to 29000 in 2000 to currently more than 85000 (Gilard et al., 2015). For example, approximately 38 million adults in the US (18.9% of the population) use herbs or other natural supplements for a variety of reasons such as improving or maintaining overall health, balancing the diet, perking up their appearance, boosting performance or delaying the onset of age-related diseases, but rarely under their physician's or pharmacist's advice. Only every third of these individuals tells their physician about this use (Kennedy et al., 2008; Wu et al., 2012). In Europe, similar trends have been observed.

The increasing popularity of NPs is based on the sometimes dangerous assumption that natural means safe (WHO, 2004), and assumption that herbal and food supplement products must be effective and safe because they are "pure" and "natural" (Stickel and Shouval, 2015). The belief that NPs are safe and free from adverse effects is simply false. Plants contain hundreds of constituents and some of these compounds are very toxic, such as the most cytotoxic anti-cancer plant-derived drugs, digitalis and pyrrolizidine alkaloids (Calixto, 2000).

Since herbs are mainly regarded and marketed as food supplements, they are not scrutinized and regulated as conventional medicines (or even as over-the-counter medicines). Manufacturers are thus exempted from pre-marketing efficacy and safety testing of a herbal product and from post-marketing surveillance. Thus, the active constituents, purity, efficacy and safety of herbal products are often unknown. **In conclusion, a variety of factors complicate the use of NPs, such as lack of scientific evidence of their safety and efficacy, lack of regulatory supervision, lack of quality control and lack of knowledge about herb-drug interactions among patients and health care providers as well as under-reporting and underestimation of adverse effects** (Shi and Klotz, 2012). In addition, it is known that there may be substantial batch-to-batch variability in NP compositions, because this will depend on plant growing locations, harvesting techniques, storage and decoction preparation (Pelkonen et al., 2014). In addition, active or toxic ingredients may be different when plants are harvested at different seasons or extracted with variable methods. Furthermore, contamination of NPs with pesticides, fungal toxins, microorganisms, heavy metals, and even synthetic compounds or drugs has been described (Efferth and Kaina, 2011; Gilard et al., 2015; Stickel and Shouval, 2015).

Due to the complexity of NPs, quality control, standardization and stability studies are difficult to undertake, and regulation of these drugs is not standardized in different countries. Guidelines for good manufacturing practice (GMP) and good agricultural practice (GAP) and use of reference reagents should be used routinely for standardization

of manufacturing of NPs. However, regulatory control with these procedures is still not enforced universally. Because of these complex characteristics, there are significant analytical challenges and a clear need for validated and publicly available methods for NP analysis. This is an ongoing process, and systematic evaluations of botanical products are still in their infancy (Calixto, 2000; Betz et al., 2007; Sahoo et al., 2010; Furst and Zundorf, 2015; Stickel and Shouval, 2015). However, the rapidly expanding use of NPs, especially those used as herbal food supplements, has focused the attention of the scientific community and regulatory authorities on the quality and safety assurance of such compounds (Stickel and Shouval, 2015).

Despite the current popularity of herbal medicines, the clinical evidence in support of the use of most herbal medicines is weak. The lack of controlled and randomized clinical trials of herbal medicines with a long history of use is the rule rather than the exception, and many of them have not been proven as either safe or efficacious by today's Western medicine standards. "The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its use worldwide", was claimed in the WHO report in 2000 (General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, available at Internet: http://apps.who.int/medicinedocs/en/d/Jwhozip42e/). The reasons for the lack of scientific research data on traditional medicines are due not only to health care policies, but more importantly due to a lack of adequate or accepted research methodology for evaluating traditional medicine. This is important to be borne in mind also when considering NPs (herbal medicinal products and food supplements). It is important to note that there are both published and unpublished data on research in traditional medicine in different countries, but more research still needs to be conducted to evaluate their safety and efficacy, in particular, the research quality should be improved. Thus, as a prerequisite, there is a need for new relevant and reliable research methods. The reductionistic approach, behind the development and use of conventional single-component medicines, is not appropriate for multi-component medicines, and new scientific approaches, more global systems biology approaches would be needed (Kell, 2013; Pelkonen et al., 2014; Vasange, 2014).

2.1.5 Regulation of natural products and evidence-based phytotherapy: Where do we stand?

The regulatory status of NPs ranges from low authorization requirements to complex legislation. Thus, the pharmaceutical quality of NPs can vary from analytically uncontrolled mixtures to high-tech specialized extracts. Herbal preparations have not been acknowledged previously as medicines; not only is this probably one of the reasons why their efficacy and adverse effects are often underestimated but it also might have contributed to unintentionally supporting the assumption that natural means safe. In addition, due to the increasing popularity of NPs, there have been increasing concerns that there are inadequate controls on their sale. A number of studies in recent years have highlighted the potential risk inherent in the uncontrolled use of NPs concurrent with conventional therapeutic regimens and emphasized the need for regulation in this field (Sahoo et al., 2010; Pelkonen et al., 2014; Furst and Zundorf, 2015).

There are a number of herbal ingredients that have accepted usage in a range of different categories. Botanical products marketed in Europe are diverse and can be sold under different classifications, classified as herbal medicinal products, food supplements, cosmetics, foods and beverages depending on the relevant applicable legislation.

'**Medicinal products**' are defined in Directive 2001/83/EC as: "(a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or (b) Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis".

'**Food supplements**' are defined in Directive 2002/46/EC as: "foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities". In addition, "there is a wide range of nutrients and other ingredients that might be present in food supplements including, but not limited to, vitamins, minerals, amino acids, essential fatty acids, fibre and various plants and herbal extracts."

Thus, the presence of substances with a nutritional or physiological effect and the form (such as capsules, pastilles, tablets, etc) result in a presentation of products that closely resembles that of medicinal products. **Consequently the form and composition per se cannot be utilized to resolve if a product should be considered as a medicinal product or a food supplement.** Thus, the borderline between medicinal products and food supplements is especially vague as **both products are intended to be ingested in a dosed form, can be presented in the same form and contain similar ingredients** often used in both categories as bioactive substances or botanicals. In many cases, the distinction between medicinal products and food supplements can easily produce confusion and mislead the consumers. Food supplements can be bought over-the-counter in pharmacies, supermarkets, specialty shops and through the Internet.

In the EU, the classification of a product is made by the responsible company – not by the authorities – based on the intended use of the product, and the requirements and consequences and restrictions for companies differ dramatically depending on the relevant applicable legislation. Currently, there is no EU legislation specifically targeting botanicals, other than the general EU food legislation. The European Medicines Agency (EMA) works currently also with the herbal medicinal products but not in the areas of food or cosmetics. The intention of pharmaceutical legislation is primarily the protection of human health and this it encompasses both the pre-marketing licensing procedure and post-marketing pharmacovigilance. (Pharmacovigilance is the science and practices relating to the detection, evaluation, understanding and prevention of adverse reactions and other medicine-related problems of marketed drugs (Jeetu and Anusha, 2010).) The basic rule is that all medicinal products need a marketing authorization if they are to be placed on the EU market. The pre-marketing licensing procedure allows and requires authorities to confirm the safety, quality and efficacy of a product that is presented or intended for the therapy or prevention of diseases. Medicinal products are launched after the regulatory authorities have scrutinized a massive amount of scientific data from animal and clinical

studies and found it to demonstrate that the drug is adequately effective and safe for the intended indications. The real safety profile of a medicinal product is characterized by strict post-marketing surveillance through a spontaneous adverse event monitoring system and a post-marketing monitoring/non-interventional studies, so called post-authorization safety (PAS) studies (Suvarna, 2010; Borg et al., 2015).

Recently, the European legislation on medicinal products also addressed the medicinal use of products that are made from substances from plants (botanicals), and are now known as "herbal medicinal products" (HMPs). Two categories are defined: i) herbal medicinal products (HMPs) (kasvirohdosvalmiste in Finnish) can be granted a marketing authorisation; and ii) traditional herbal medicinal products (THMPs) (perinteinen kasvirohdosvalmiste) can be granted a registration license based on their longstanding use if they are complying with a set of provisions ensuring their safe use. Hence, nowadays herbal preparations are divided either into medicines or food products. The European Directive 2004/24/EC released in 2004 (with a 7 years' transitional period that ended on 30 April 2011) by the European Parliament and by the Council of Europe provides the basis for the use of herbal medicines in Europe in the future. The goal of the legislation is to confirm the existence of such products and to consider particular certain characteristics when evaluating quality, efficacy and safety.

Hence, since 2004, in Europe, companies can apply for three different types of market authorization of an herbal medicinal product (Table 2). The first type is **full application**, that means that the manufacturer must verify efficacy and safety in their own studies and is identical to the application of a NCE. The second type is **Well-established medicinal use (vakiintunut lääkinnällinen käyttö in Finnish)**. A manufacturer can obtain an approval by showing, on the basis of a detailed scientific bibliography, that the HMP has been in medicinal use for at least 10 years in Europe and has recognized efficacy and an acceptable level of safety. The third type is **Traditional use**, when the product will be registered. Not less than 30 years for medicinal use, with at least 15 years use in Europe, needs to be documented and efficacy must be plausible on the basis of the long time of experience. By law, these HMPs have a specified, and generally a low, dose.

Table 2. Three different types of herbal medicinal product market authorization (Furst and Zuendorf 2015).

	Full application	**"Well-established medicinal use" (since 2002)**	**"Traditional use" registration (since 2004)**
Pharmaceutical quality	must be demonstrated	must be demonstrated	must be demonstrated
Time of usage	-	used for ≥ 10 years in Europe	used for ≥ 30 years including ≥ 15 years in Europe
Efficacy	preclinical and clinical data	based on a detailed scientific bibliography	must be plausible on the basis of experience
Safety	preclinical and clinical data	based on a detailed scientific bibliography	bibliographic overview
Annotation	-	-	defined dosages and applications against mild health disturbances

The main difference between medicinal products (full applications) and HMPs and THMPs (well-established and traditional use) is the requirements concerning scientific data, i.e., scientific studies to verify the safety, quality and efficacy. The HMPs and THMPs are accepted and registered if they fulfil the requirements of well-established medicinal use or long time of usage based on bibliography, not based on scientific studies planned especially for certain substances/products to demonstrate their efficacy, quality and safety – as is the case with conventional medicines. It is noteworthy that performing experimental studies and clinical trials with plant extracts can encounter some pitfalls. For example, it is not possible to investigate the efficacy of St. John's wort per se, but only of a certain (hopefully well characterized) extract of the plant. Thus, studies on "St. John's wort" can only be compared when exactly the same extract has been used (Furst and Zundorf, 2015).

It is useful to keep in mind that even if the guidances and regulation are strengthened to the same level as regulated medicines, the lack of a preapproval review process (as well as pitfalls in the scientific studies) for all supplements will continue to limit the regulatory authorities' ability to ensure that these food supplements are safe.

2.2 XENOBIOTIC METABOLISM AND DRUG METABOLISM

The human body processes all xenobiotics similarly, thus xenobiotic metabolism and drug metabolism can be considered as synonyms.

Drug metabolism is a biochemical process in which lipophilic drugs are transformed into more water soluble metabolites, facilitating their elimination from the body and terminating their effects. To a large extent, metabolism determines the pharmacokinetic behavior of a drug, i.e., the intensity and the duration of action, affecting both the compound's efficacy and safety. In addition to the protective detoxification function of drug metabolism, metabolites may have also pharmacological or even toxicological properties, affecting the clinical profile of the drug. Thus, drug metabolism is related to both efficacy and safety of a drug, which are two key elements associated with successful drug therapy. Overall, drug metabolism has an impact on drug action, adverse effects, interactions and interindividual differences, all matters of great importance in drug treatment (Pelkonen et al., 1998; Gonzalez et al., 2011).

Drug metabolism is divided into two categories, phase I (functionalization) and phase II (conjugation) enzymatic transformation reactions. Phase I reactions include oxidation, hydrolysis and reduction, introducing or exposing a functional group in the drug molecule, such as hydroxyl (–OH), amino ($–NH_2$), carboxyl (–COOH), thiol (–SH) or epoxide. This functional group is subsequently conjugated with some water soluble moiety, such as glucuronic acid, sulfate, glutathione (GSH) or glycine in phase II metabolism. With a very few exceptions, all xenobiotics are subjected to one or multiple pathways that constitute the phase 1 and 2 enzymatic systems. The principal site of drug metabolism is the liver, which contains the vast majority of metabolizing enzymes. A majority of crucial steps within drug metabolism are conducted by the CYP enzymes, which represent the most important enzyme system in phase I metabolism (Pelkonen et al., 1998; Iyanagi, 2007; Testa et al., 2012).

2.3 CYP ENZYMES

CYP enzymes comprise a superfamily of heme-containing monooxygenases involved in the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds, such as steroids, prostaglandins, bile acids, drugs, chemical carcinogens and NPs. CYP enzymes are the predominant enzymes involved in human drug metabolism. It is generally thought that about 90% of all phase I metabolism of xenobiotics is carried out by the of CYP enzymes, and since around 75% of all drug metabolism (both phase I and phase II) is mediated by CYP enzymes, it is clear that these enzymes are of considerable importance in the way that chemicals are cleared by the body. CYP enzymes are most abundantly expressed in the liver, which is the main organ for drug metabolism, but they are also located in extrahepatic tissues, in the intestine, skin, lungs and kidneys (Guengerich, 2008; Zanger et al., 2008; Lewis and Ito, 2009; Zanger and Schwab, 2013).

The human genome contains a superfamily of 57 CYP proteins, of which 16 enzymes in families CYP1, 2 and 3 are responsible for oxidative and reductive metabolism of most drugs (~75%) as well as the broad and variable range of xenobiotics to which an individual is exposed (Table 3). In contrast, enzymes in families CYP4 to CYP51 are responsible for the synthesis or processing of steroids, fatty acids, eicosanoids and vitamins or have functions

that are not yet understood (Table 3). The CYP enzymes primarily devoted to xenobiotic metabolism have broad and overlapping substrate specificities, in accordance with the fact that a small number of enzymes must cope with a potentially very large and diverse chemical structures. An individual xenobiotic-metabolizing CYP may oxidize hundreds of structurally different substrates, while those involved in endogenous functions often metabolize a few similar substrates. The CYP enzymes involved in biosynthetic processes have narrow substrate specificities as they are intended to process certain specific endogenous substrates, e.g., 14α-demethylation of lanosterol (CYP51) (Debeljak et al., 2003), cholesterol side chain cleavage (CYP11A1) (Storbeck et al., 2007), and aromatization of testosterone (CYP19A1) (Simpson, 2000). Thus, specific inhibitors for aromatase (CYP19A1), such as anastrozole, have been developed for use in the treatment of estrogen-dependent tumors (Narashimamurthy et al., 2004). Relatively specific CYP enzymes are also involved in the production of eicosanoids and degradation of fatty acids (Capdevila et al., 2005; Hardwick, 2008).

Table 3. The 57 human CYP enzymes (Ortiz de Montellano, 2012).

Drug Metabolism		**Fatty Acids and Eicosanoids**	**Unknown (Orphan)**		**Sterols and Vitamins**	
1A1	2D6	2J2	2A7	20A1	2R1	24A1
1A2	2E1	4A11	2S1	26C1	7A1	26A1
1B1	2F1	4B1	2U1	27C1	7B1	26B1
2A6	3A4	4F2	2W1		8B1	27A1
2A13	3A5	4F3	3A43		11A1	27B1
2B6	3A7	4F8	4A22		11B1	39A1
2C8		4F11	4F22		11B2	46A1
2C9		4F12	4V2		17A1	51A1
2C18		5A1	4X1		19A1	
2C19		8A1	4Z1		21A2	

The broad substrate specificity of enzymes in families CYP1, CYP2 and CYP3 means that these enzymes can only exert limited control over both the types of substrates and the sites on those substrates that will be oxidized. It is therefore not surprising that CYP enzymes, in the process of oxidizing compounds to more polar and excretable metabolites, sometimes convert a relatively safe molecule into chemically reactive and potentially toxic products (e.g., procarcinogens) and chemically inactive molecules into pharmacologically active metabolites (e.g., prodrugs). A given substrate may undergo multiple CYP oxidations by one or several CYP enzymes and at different positions on the molecule. In addition, CYP catalyzed reactions can occur even at positions that are chemically difficult to oxidize (Nebert and Russell, 2002; Ortiz de Montellano, 2012).

Among this large family of oxidizing enzymes, CYP3A4 is recognized as the main enzyme involved in drug metabolism in the liver and also in the gut. The nine most important human liver CYP forms involved in drug metabolism and their proportional amounts in human liver (per cent) are as follows: CYP1A2 (~15%), CYP2A6 (<5%), CYP2B6 (<5%),CYP2C8 (<5%), CYP2C9 (~20%), CYP2C19 (<5%), CYP2D6 (~5%), CYP2E1 (~10%) and

CYP3A4/5 (~30%). CYP enzymes 1A2, 2C9, 2C19, 2D6 and 3A4/3A5 participate in the metabolism of approximately 80% of marketed drugs, with more than half of these agents being metabolized by the CYP3A family (Breimer and Schellens, 1990; Pelkonen et al., 1998; Guengerich, 2003; Orr et al., 2012).

2.3.1 History and nomenclature

CYP enzyme(s) were first found by Klingenberg in 1958 and later reported by Omura and Sato in 1962 (Klingenberg, 1958; Omura and Sato, 1962). The membrane-bound and hydrophobic nature of the mammalian CYP enzyme system (localized predominantly in the smooth endoplasmic reticulum) impeded purification, and CYP was thought to be one single enzyme in the early 1960s. By the middle 1960s, it was associated with drug and steroid metabolism and by the late 1970s, as many as six CYP enzymes were speculated to exist. In 1979, cimetidine, a histamine H2-receptor antagonist, was associated for the first time with inhibition of drug-metabolizing enzymes in rat and human liver and in human beings *in vivo* as well as drug interactions (Pelkonen, 2015). CYP2C19 enzyme, for example, was characterized in the 1990s by Wrighton et al. (1993) and by Goldstein et al. (1994) by demonstrating that the (S)-mephenytoin hydroxylase is CYP2C19. Later, polymorphism of the *CYP2C19* gene and its clinical relevance were elucidated, as reviewed by Goldstein (2001).

These heme-thiolate proteins are named by a unique spectral absorbance peak at 450 nm when reduced and bound to carbon-monoxide (CO) (Nebert and Russell, 2002): cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm. The absorbance band at 450 nm is unique to heme proteins and serves as a signature for active CYP proteins. The spectroscopic properties of all CYP enzymes are similar and can be used to monitor the binding of substrates to the enzymes (Estabrook, 1996).

CYP enzymes are found throughout nature, in mammals, fungi, bacteria, insects and plants. Over 12000 CYP genes have been identified. Plants have particularly high numbers of CYP genes. CYP enzymes constitute the largest family of enzymatic proteins also in plants acting on various endogenous and xenobiotic molecules (Rasool and Mohamed, 2015). Although the CYP folding is highly conserved, there is enough structural diversity to permit the binding of substrates of significantly different sizes to different CYPs and with varying degrees of specificity (Denisov et al., 2005).

The CYP superfamily is organized into families and subfamilies based on their structural correlations, i.e., amino acid sequence identities, which uniquely identifies each of the CYP enzymes (Nebert et al., 1987; Nelson, 2006). Enzymes that share ≥ 40% amino acid sequence identity are grouped within the same family and enzymes that share more than 55% identity are placed into the same subfamily. In practice, CYP enzymes are assigned a number designating the family, a letter indicating the subfamily and a second number that identifies the individual protein (see Table 3, page 21). Terms P450 and CYP are used in parallel and as shorthand notations for cytochrome P450. Thus, both P4503A4 and CYP3A4 refer to the fourth enzyme of subfamily A in family 3. The name of the gene coding for the protein is the same as the CYP form, except that it is given in italics, i.e. *CYP3A4*. Each enzyme is termed an isoform since each derives from a different gene. Since function

usually follows overall structure, this sequence-based nomenclature system provides a general grouping of enzymes with relatively similar substrate selectivities. However, the structural similarity of enzymes cannot be used to predict which isoforms will be responsible for a particular xenobiotic's metabolism. Although there are examples known whereby CYP enzymes in different subfamilies and even families metabolize the same substrate, the region- and stereoselectivity of metabolism is often significantly different in these cases. This nomenclature system facilitates recognition of the analogies between enzymes of the same or different species (Ortiz de Montellano, 2012).

2.3.2 Structure and function

X-ray crystallography techniques have become a major tool for determination of three-dimensional crystalline structures on the atomic scale. Based on the crystal structures, it has been possible to draw conclusions regarding the overall structural characteristics of CYP enzymes. The first three-dimensional bacterial CYP crystal structure was described in 1985 (Poulos et al., 1985), and the first mammalian (CYP2C5) and the first human (CYP2C9) CYP crystal structures were resolved in 2001 and 2003, respectively (Tennant and McRee, 2001; Williams et al., 2003; Wester et al., 2004). Recently, the dream of obtaining crystal structures of the principal human drug-metabolizing CYP enzymes has become a reality and they have also been made available in Protein Data Bank (PDB) (http://www.pdb.org/) (Berman et al., 2002). The structures of normally membrane-bound mammalian proteins (localized predominantly in the smooth endoplasmic reticulum) have the same general fold (i.e. aspect/appearance) as those of the soluble bacterial CYP enzymes.

Human CYP enzymes are composed of about a 500 amino acid polypeptide chain and a heme-iron center (iron protoporphyrin IX) as a prosthetic group (Figure 5) (Kirchmair et al., 2012). The heme moiety acts as an oxidation reaction center and the apoprotein regulates the substrate specificity and binding affinity of individual CYP enzymes (Guengerich, 2001; Yan and Caldwell, 2001; Denisov et al., 2005). Above and below the plane of the heme, there is room for "fifth" and "sixth" ligands (four ligands are provided by nitrogens on the heme ring). The central iron atom of the heme group is bound noncovalently to the sulfur atom (S^-) of a cysteine residue (acting as the fifth thiolate ligand) situated vertically near the heme, close to the C terminus of the apoprotein chain. Cysteine forms a thiolate bridge with the heme iron producing the characteristic Soret absorbance peak at wavelength 450 nm and being essential to the catalytic activity of the enzyme. The sixth ligand is often a water molecule that substrate binding will replace. The iron atom of the heme prosthetic group can occur in two oxidation states, Fe^{2+} (reduced) and Fe^{3+} (oxidized).

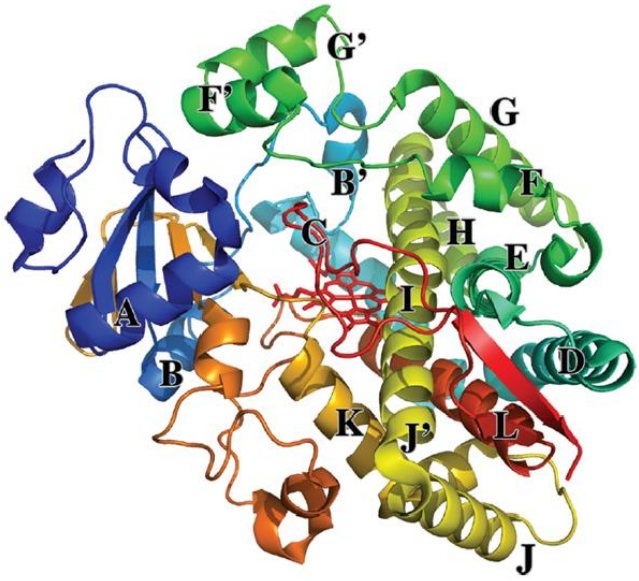


Figure 5. Structural overview of CYPs is depicted here using CYP3A4 (PDB 3NXU) as a template. Helical structures are labeled A–L according to the general scheme for CYPs and the heme moiety is indicated in sticks mode with red color (Kirchmair et al., 2012).

CYPs exhibit a conserved secondary structure and folding pattern, mainly with four β-sheets and approximately 13 α-helices. Although the CYP fold is greatly conserved, there is sufficient structural diversity to allow for the binding of substrates of significantly different sizes to different CYPs. Their most conserved structural features are related to heme binding and some common catalytic properties (Werck-Reichhart and Feyereisen, 2000; Guengerich, 2001). Well-conserved α-helices are designated by letters A to L beginning at the N-terminus of the catalytic domain as originally defined for the first CYP structure (Poulos et al., 1985), whereas sheets and component strands are identified numerically in the same fashion. Helices F and G are above the active site and the heme prosthetic group lies between the I and L helices. The residues of the I helix near heme have been proposed to be important for catalytic activity of CYPs. Generally, the closer to the heme, the more conserved the structure, particularly in helices I and L, which make direct contact with the heme. As expected, those regions which control substrate specificity differ the most, especially the B' helix. Six substrate binding regions are recognized that line the active site (Gotoh, 1992). In particular, these regions, related to helices B, F, G, I and K, significantly affect substrate specificities, and predetermine CYP substrate specificity.

Conformational adaptations also play a role in the capabilities of mammalian drug and xenobiotic metabolizing CYP enzymes to recognize structurally varied substrates and multiple oxidation sites are frequently seen for substrates (Poulos and Johnson, 2005). This is likely to affect either the ability of the active site to bind the substrate in more than one orientation and/or location, or to allow the motion of substrate within the active site cavity. From a structural perspective, there is probably the greatest understanding of specific small-molecule ligand interactions with CYP enzymes obtained mainly from numerous X-ray crystallography structures (i.e., the X-ray structures of human CYP enzymes co-crystallized with various ligands). It has become apparent that the flexibility of several

xenobiotic-metabolizing CYP active sites results in highly different interactions with different ligands. The flexibility of these CYP active sites can limit the predictive value of one ligand structure for other ligands. This conformational flexibility associated to substrate accommodation poses considerable difficulties in predicting substrate selectivity from a perusal of the X-ray structure of a CYP enzyme. Thus, X-ray structures in themselves do not automatically provide information about function. It is commonly appreciated that the structure of a specific CYP-ligand complex is of limited utility in understanding the binding mode(s) of structurally different ligands to the same CYP. Existing knowledge does not provide sufficient information to allow us to fully understand the rules that determine the nature of CYP/ligand interactions. The size and shape of the CYP active site are currently believed to be the key determinants for substrate selectivity. The number of hydroxyl groups, stereostructure, molecular weight and lipophilicity, for example, seem to exert some sort of effect on individual results. Thus, understanding CYP/ligand interactions and making predictions of structure/function relationships are major challenges (Dong et al., 2012; Johnson et al., 2014). However, the recent study by Sevrioukova and Poulos (2017) highlights new aspects of CYP3A4 conformational adaptation and function with midazolam (i.e. the most specific substrate and the best *in vivo* probe for the CYP3A4 activity prediction) and effector molecules such as α-naphthoflavone (ANF). Midazolam was observed to trigger structural changes both within and far away from the CYP3A4 active site and ANF could modulate the drug metabolism by associating with the affected areas and changing conformational dynamics. Several surface clefts in the midazolam-bound CYP3A4 were detected where ANF and other effector molecules could potentially bind.

CYP enzymes are claimed to catalyze more than 60 different types of chemical reactions, for example N- and S-oxidations, aromatic and aliphatic hydroxylations, dealkylations and epoxidations (Estabrook, 1996; Guengerich, 2001). The primary catalytic function of these monooxygenases is to achieve the incorporation of a single atom of molecular oxygen (O_2) into a substrate with the simultaneous reduction of the other atom to water (Makris et al., 2005; Ortiz de Montellano and De Voss, 2005). This catalytic process requires the transfer of electrons from a reducing cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to the CYP enzyme. The molecular oxygen activation and oxygen atom insertion into the substrate are mediated by the heme iron atom. The overall reaction catalyzed by CYP enzymes has the following stoichiometry and can be written as below, where RH is a substrate and ROH is the product formed by addition of an oxygen atom to it:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$ (Bernhardt, 2006).

In mammalian systems, this catalytic process requires the transfer of electrons from NADPH to the CYP enzyme by NADPH-cytochrome P450 reductase (CPR) and/or cytochrome *b5*. The catalytic cycle of CYP consists of at least 7 separate steps (Figure 6):

1) binding of the substrate to the ferric (Fe^{3+}) form of the enzyme;
2) reduction of the heme group from the ferric to the ferrous (Fe^{2+}) state by an electron provided by NADPH via CYP reductase;
3) binding of molecular oxygen;
4) transfer of a second electron from CYP reductase and/or cytochrome b5;
5) cleavage of the O–O bond;

6) substrate oxygenation, and
7) product release.

Although impairment of any of these steps can lead to inhibition of CYP enzyme activity, steps (1), (3) and (6) are particularly vulnerable to inhibition (Lin and Lu, 1998; Yan and Caldwell, 2001; Guengerich, 2013).

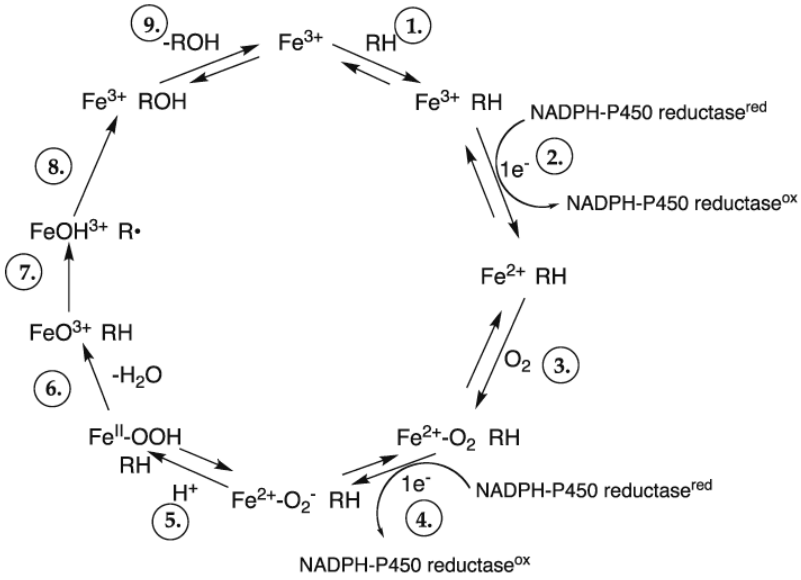


Figure 6. Generalized catalytic cycle for CYP reactions. **Fe**, iron atom in CYP heme; **RH**, drug/substrate; **ROH**, product/oxidized drug; **NADPH**, reduced nicotinamide adenine dinucleotide phosphate; **NADP$^+$**, nicotinamide adenine dinucleotide phosphate; **e$^-$**, electron; **^{ox}** and **^{red}** indicate the reduced and (1-electron) oxidized states of the reductase involved in the electron transfers (Guengerich, 2013).

2.3.3 Spectroscopic properties of CYP enzymes

The spectroscopic properties of the heme group provide important information on the CYP enzyme active site. The spectroscopic properties of the heme group are sensitive to the nature of the heme iron ligands, i.e. to the oxidation state of the iron atom and the polar and hydrogen-bonding nature of the environment around the heme (Schenkman et al., 1982; Ortiz de Montellano, 2012). Spectral interaction studies have been conducted from the early years of CYP research. Compounds that have affinity to an active site of a CYP coordinate towards the heme iron, which causes a change in the spin equilibrium of heme iron. These spectroscopic changes are highly valuable, since they make it possible to spectroscopically monitor the binding of inhibitors and substrates to CYP enzymes.

The CYP enzyme family can be differentiated from practically all other hemoproteins by the UV–vis spectrum of their ferrous–CO complexes. The intense Soret absorption maximum of this ferrous–CO complex appears at ~420 nm for most hemoproteins, while in CYP enzymes, it is split into two peaks with maxima at ~380 and ~450 nm. The peak at ~450 nm is taken as the specific hallmark of the CYP enzymes and is the source of the name for

these proteins. This unique and characteristic absorbance fingerprint identifying a CYP is due to the coordination of a thiolate ligand (RS^-) of cysteine to the heme iron of the hemeprotein, a fact that also contributes to its unique properties as an "oxygen-activating" enzyme. The carbon monoxide spectrum forms the basis for the quantitative estimation of CYP enzymes. One of the first indications that a CYP enzyme is denatured is a shift of the 450-nm ferrous–CO spectrum to ~420 nm. This shift indicates that the proximal thiolate ligand has been protonated to a thiol ligand or has been fully displaced from the iron by another functionality (Perera et al., 2003).

The ferrous–CO spectrum of CYP enzymes defines their spectroscopic signature, as mentioned above, but the UV–vis spectra of the ferric (Fe^{3+}) enzymes are also informative. The most important optical difference spectra of oxidized (Fe^{3+}) CYP enzyme are: type I, with an absorption maximum at 385–390 nm and a minimum around 420 nm: and type II, with a peak at 420–435 nm and a trough at 390–410 nm (Figure 7). The two most important optical difference spectra of the reduced (Fe^{2+}) CYP enzyme are: the carbon monoxide spectrum, with its maximum at or about 450 nm: and the type III spectrum, with two pH-dependent peaks at approximately 430 and 455 nm.

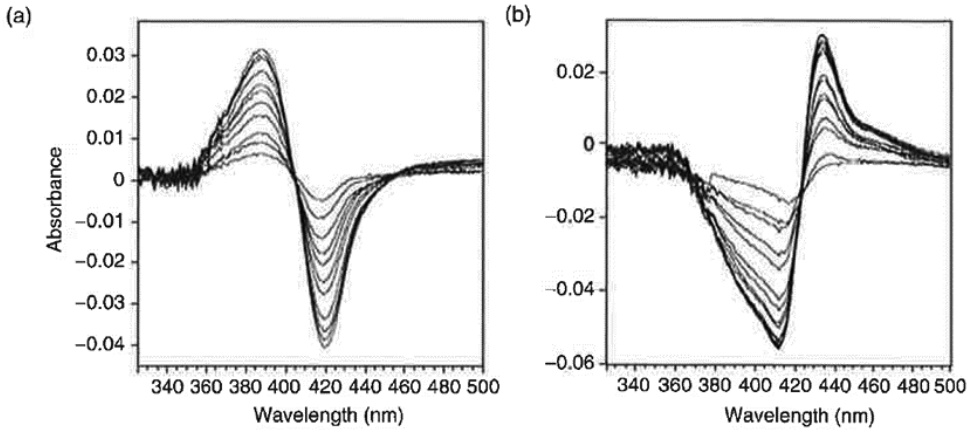


Figure 7. Typical difference spectra for the binding of ligands to a CYP enzyme (with different ligand concentrations) giving: a) a type I difference spectrum, and b) a type II difference spectrum (Ortiz de Montellano, 2012).

When a distal axial water ligand is present, the CYP iron atom exists in the low spin state and the CYP protein has a Soret absorption maximum at ~416–419 nm. Displacement of this distal water ligand, i.e., leaving the sixth iron coordination site vacant, gives rise to a high spin iron with a Soret absorption maximum at 390–416 nm. CYP enzymes can exist as an equilibrium mixture of low and high spin states, but mostly favoring the low spin state. When the distal water ligand is present, its displacement by a substrate causes a shift in the absorption maximum from ~419 to 390 nm. The resulting difference spectrum is known as a type I binding spectrum (Schenkman et al., 1982). Exemptions to this substrate-dependent spectroscopic shift occur if the resting enzyme does not have a distal water ligand, as is the case with CYP1A2 and CYP2D6 enzymes (Rowland et al., 2006; Sansen et al., 2007), or, occasionally, if the binding of a compound does not displace the distal water ligand.

Type I ligands exist in many different chemical classes. They appear to bind to a hydrophobic site in the CYP protein that is close enough to the heme allowing both spectral

perturbation and interaction with the activated oxygen. Although type I ligands are generally substrates, it has not been possible to reveal a quantitative relationship between *K*s (the concentration required for half-maximal spectral development) and K_m (the Michaelis-Menten constant) (Ortiz de Montellano, 2012).

A different spectroscopic shift is observed if the added ligand coordinates strongly to the heme iron atom; type II ligands interact directly with the heme iron of the cytochrome and are generally organic compounds having nitrogen atoms with sp^2 or sp^3 non-bonded electrons that are sterically accessible. For instance, a type II binding spectrum is detected when the nitrogen of an imidazole or triazole ring binds to the CYP heme iron atom. Many type II ligands are inhibitors of CYP-dependent monooxygenase activity (Ortiz de Montellano, 2012).

The best-known type III ligands for CYP enzyme are ethyl isocyanide and methylenedioxyphenyl (MDP) compounds such as the insecticide synergist piperonyl butoxide that, upon metabolism form stable type III complexes that appear to be related to the mechanism by which they inhibit monooxygenase reactions (Hodgson et al., 2015). The resulting ferrous (Fe^{2+}) complex is characterized by a difference absorption spectrum with maxima at 427 and 455 nm, whereas the ferric (Fe^{3+}) complex exhibits a single absorption maximum at 437 nm. The ferrous peaks at 427 and 455 nm are a result of separate complexes, although their structural interrelationship is unclear (Wilkinson et al., 1984; Correia and Ortiz de Montellano, 2005; Hodgson et al., 2015).

2.4 CYP POLYMORPHISMS

Individual variability in drug safety and drug efficacy is a major challenge in current clinical practice and drug development. The effects of many drugs, both beneficial and harmful, may differ widely from one patient to the next. The recognition that a part of this variation is inherited, and thus predictable, created the field of pharmacogenetics over 50 years ago (Meyer, 2004).

Drug metabolism varies considerably from one individual to the next, due to genetic, physiological, pathophysiological and environmental factors, which can affect the outcome of drug therapy. One of the main causes of interindividual variation of drug effects is genetic variation in the drug-metabolizing enzymes. The most common reasons for genetic variation are genetic polymorphisms, attributable to the presence of mutations in the genes for these enzymes, especially single nucleotide polymorphisms (SNPs). This can cause increased, decreased or absent enzyme expression or activity by numerous molecular mechanisms. Polymorphisms produce different subgroups in the population that differ in their ability to perform certain drug biotransformation reactions. The degree to which genetic polymorphism plays a role in determining expression and function is different for each individual *CYP* gene. Four potential CYP phenotypic subgroups exist: the ultrarapid metabolizers (UM) (carrying multiple functional genes), the extensive metabolizers (EM) (carrying two functional genes), the intermediate metabolizers (IM) (carrying one functional and one defective gene) and the poor metabolizers (PM) (lacking the functional CYP enzymes) (Meyer and Zanger, 1997; Ingelman-Sundberg et al., 2007; Zanger et al., 2008; Zanger, 2012).

The CYP1–CYP3 family isoenzymes (i.e., drug-metabolizing CYP enzymes) show extremely variable expression and function, typically exceeding 100-fold within a given population sample, which leads to unexpected drug responses; these can range from a lack of response to an overreaction or even overt toxicity in a substantial fraction of patients who have been treated with drug substrates of these enzymes. However, although genetic polymorphisms are present in practically all human genes, they affect only some CYP isoforms to a functionally relevant extent. In particular, these involve CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and the minor CYP3A forms 3A5 and 3A7. In contrast, the highly abundant liver CYP enzymes 1A2, 2C8 and 3A4 are not functionally polymorphic since the known sequence variants only marginally affect their expression or function (Zanger, 2012).

CYPs 1A2, 2C8 and 3A4, which lack major functional polymorphisms, are known to be responsible for the metabolism of about half of all drugs. This means that the polymorphic CYPs 2B6, 2C9, 2C19 and 2D6, which have an established role in pharmacogenetics, are responsible for the metabolism of the other half of marketed drugs (Meyer, 2004; Zanger et al., 2008). In addition, it has been reported that almost half of the drugs causing adverse drug reactions are metabolized by polymorphic CYP enzymes (Singh et al., 2011). Knowing the gene variants that are responsible for differences among patients has the potential to allow so-called personalized drug therapy (e.g., by adjusting the dosage) to avoid therapeutic failure and serious adverse effects. For example, codeine (an alkaloid) is a prototypical "prodrug" in that its analgesic effect is almost totally dependent on its biotransformation to morphine. This process is mediated by the polymorphic CYP2D6. Consequently, interindividual variability in codeine metabolism and response is a clinical reality, having potential for both lack of therapeutic effect and life-threatening adverse reactions – although the therapeutic window for codeine is not narrow per se (since there is a wide variability in "safe" therapeutic dosing) (Kelly and Madadi, 2012).

The CYP2C19 genetic polymorphism is now relatively well understood and some drugs are known for which the variant 2C19 phenotype is a problem. The phenotype of CYP2C19 metabolic capacity can be categorized as EMs, IMs and PMs. The majority of *CYP2C19* pharmacogenetic studies have been conducted using variants exhibiting the PM phenotype, especially *CYP2C19**2, *3, and *17. The greatest uncertainty is in the IM group, in which interindividual variation is clearly observed but the underlying mechanism for this variation remains unclear (Lee, 2012). Approximately 2% of Caucasians, 4% of African Americans, 14% of Chinese and 20% of Asians carry two loss-of-function variant alleles, classified as PMs since they exhibit little or no CYP2C19 enzyme activity. On some Pacific islands, the incidence of PMs is as high as 75% (Nakamura et al., 1985; Blaisdell et al., 2002; Dean, 2012; Amin et al., 2017).

Reliable phenotyping and genotyping tools are available for screening CYP2C19 activity in patients, but these are not routinely used in clinical practice to design appropriate therapeutic regimens and improve the outcome of treatment with CYP2C19 substrate drugs (Desta et al., 2002; Amin et al., 2017). Clopidogrel is an example of a drug for which *CYP2C19* genotyping is believed to be clinically important to improve patient outcomes and minimize patient risk. In addition to CYP2C19 inhibition, also CYP2C19 polymorphisms may lead to a reduction in clinical efficacy of the prodrug clopidogrel. Clopidogrel is bioactivated in two steps to the active metabolite, via CYPs 1A2, 2B6 and 2C19 in the first step and via CYPs 2B6, 2C9, 3A4/5 and 2C19 in the second step of its

bioactivation process (Amin et al., 2017). A recent systematic review and meta-analysis stated that carriers of *CYP2C19* loss-of-function alleles (PMs) are at a greater risk of stroke and composite vascular events than noncarriers among patients with ischemic stroke or *transient ischemic attack* (TIA) treated with clopidogrel (Pan et al., 2017). In addition, the FDA-approved drug label for clopidogrel contains a boxed warning, stating that clopidogrel has diminished effectiveness in *CYP2C19* PMs, advising to test *CYP2C19* genotype (Dean, 2012). The Clinical Pharmacogenetics Implementation Consortium (CPIC) has issued antiplatelet therapy recommendations for *CYP2C19* IM and PM patients with acute coronary syndrome who are undergoing percutaneous coronary interventions, such as the placement of a stent, to use an alternative antiplatelet agent (e.g., prasugrel or ticagrelor) when not contraindicated (Dean, 2012).

In addition, increased toxicity of substrate drugs (e.g. phenytoin and tricyclic antidepressants) in *CYP2C19* PMs or during drug interactions have been reported (Desta et al., 2002). For example, undesirable adverse effects such as prolonged sedation and unconsciousness have been observed after administration of diazepam in *CYP2C19* PMs (Bertilsson et al., 1989). Instead, proton pump inhibitor drugs have been observed to exhibit a greater cure rate for gastric ulcers with *Helicobacter pylori* infections in PMs than in EMs due to higher plasma concentrations of the parent drugs in PMs (Sohn et al., 1997; Furuta et al., 1998). In summary, the clinical decision following *CYP2C19* genotyping involves two possibilities: an adjustment of the drug dose according to the genotype or an alternative drug choice.

2.5 INHIBITION OF CYP ENZYMES

Modulation of CYP activity via inhibition or induction by xenobiotics can lead to clinical drug interactions with consequences ranging from loss of efficacy to the appearance of adverse effects with the coadministered drugs (Orr et al., 2012). Inhibition of CYP enzymes is the most common cause of metabolism-based drug interactions and of clinical importance for both therapeutic and toxicological reasons. Inhibition of CYP enzymes has resulted in the removal of numerous drugs from the market during the past years (Wienkers and Heath, 2005).

Enzyme inhibition signifies a decrease in enzyme activity, i.e., a decrease in the rate of substrate turnover due to the presence of an inhibitory compound. Enzyme inhibitors fall into two broad classes – reversible and irreversible (mechanism-based) inhibitors. Reversible inhibition most typically involves a rapid association and dissociation between inhibitors and enzymes, due to noncovalent interactions, for instance hydrophobic interactions, hydrogen bonds and ionic bonds. The sum of the various weak interactions between the inhibitor and the enzyme results in strong, specific, but still reversible binding. The enzyme activity will recover soon after the removal of the inhibitor. In contrast, irreversible inhibitors undergo a CYP-catalyzed activation (mechanism-based inhibition) that leads to enzyme inactivation through covalent or quasi-irreversible modification of the enzyme structure. After mechanism-based inhibition, enzyme activity can be replaced only by newly synthesized protein (Kramer and Tracy, 2012; Kamel and Harriman, 2013).

In order to distinguish between these two processes, and to design appropriate *in vitro* experimental conditions, an understanding of enzyme inhibition kinetics is essential. The

determination of *in vitro* CYP inhibition kinetics is a significant predictor of potential drug interactions.

The metabolism of drugs typically follows Michaelis-Menten kinetics (especially when there is only one binding site in the active site of the enzyme) as illustrated in the following scheme (Lin and Lu, 2001):

$$[E] + [S] \underset{K2}{\overset{K1}{\leftrightarrows}} [ES] \xrightarrow{k3} [E] + [P]$$

And kinetically, the velocity (v) of a reaction can be expressed with the following equation (Eq. 1), where V_{max} is the maximum velocity of metabolism and K_m is the Michaelis-Menten constant of the substrate:

Eq. 1

$$v - \frac{Vmax * [S]}{Km + [S]}$$

Under linear conditions *in vitro*, the ratio of V_{max} and K_m (V_{ma}/K_m) represents the metabolic intrinsic clearance (CL_{int}), which is assumed also to be representative of the *in vivo* CL_{int} of a compound (Lin and Lu, 2001).

2.5.1 Reversible inhibition

Reversible inhibition is probably the most common type of enzyme inhibition and can be further classified into competitive, uncompetitive or mixed-type inhibition. The competition can be either for the prosthetic heme group or for other regions in the CYP active site (Yan and Caldwell, 2001).

The most frequently observed type of reversible enzyme inhibition is **competitive inhibition**. In competitive inhibition, the binding of the inhibitor hinders binding of substrate to the active site of free enzyme. When the inhibitor is occupying the enzyme active site, the substrate is unable to bind in a productive orientation and the complex is unable to metabolize the substrate. This type of inhibition can be overcome by sufficiently high concentrations of substrate. The equation which describes competitive inhibition constants, as well as values for K_m and V_{max}, is described below (Eq. 2) (Kramer and Tracy, 2012). Competitive inhibition will result in an increase in the apparent K_m value and unchanged apparent V_{max} value (Lin and Lu, 1998; Kramer and Tracy, 2012; Kamel and Harriman, 2013).

Eq. 2

$$v - \frac{Vmax * S]}{Km\left(1 + \frac{[I]}{Ki}\right) + [S]}$$

In the case of **uncompetitive inhibition**, the inhibitor does not bind to the free enzyme, but binds only to the enzyme-substrate (ES) complex, and thus again the enzyme-substrate-inhibitor complex is nonproductive. The equation to describe uncompetitive inhibition constants is presented below (Eq. 3) (Kramer and Tracy, 2012). Because the extent of

inhibition is dependent on the amount of the ES complex and, therefore, on the amount substrate present, it increases with increased substrate concentration and is characterized by a decrease in both the apparent K_m and V_{max} values. Thus, in this case, the reduction in the effective concentration of the ES complex results in a decreased apparent K_m value and proportional decrease in the apparent V_{max}. As a result, the inhibitor has not net effect on V_{max}/K_m (i.e., enzyme efficiency or CL_{int}) (Kamel and Harriman, 2013). The uncompetitive inhibition is a rarely encountered type of inhibition.

Eq. 3

$$v - \frac{\left[\frac{V\max}{1 + \frac{[I]}{Ki}}\right] + [S]}{\left[\frac{Km}{1 + \frac{[I]}{Ki}}\right] + [S]}$$

In **mixed-type inhibition**, both competitive inhibition and uncompetitive inhibition occur. In this case, the K_m increases and the V_{max} decreases. Mixed-type inhibition can be reduced, but not completely overcome, by increasing concentrations of substrate. The effects of mixed inhibition on the velocity of the reaction can be presented by the following equation (Eq. 4) (Kramer and Tracy, 2012). Noncompetitive inhibition is a special case of mixed type inhibition having equal K_i values (Eq 4). Therefore, the substrate concentration does not affect the inhibition efficacy. The reaction will not reach the same rate, thus, V_{max} will decrease but the K_m for a probe substrate will not change (Eq. 4). Noncompetitive inhibition is rarely observed (Kamel and Harriman, 2013).

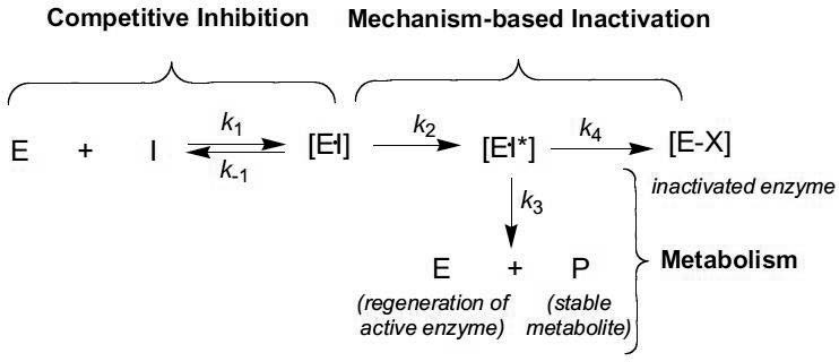
Eq. 4

$$v = \frac{V\max * [S]}{Km\left(1 + \frac{[I]}{Ki}\right) + [S]\left(1 + \frac{[I]}{Ki}\right)}$$

2.5.2 Mechanism-based inhibition (MBI)

Multiple compounds are known to undergo CYP-catalyzed activation to reactive intermediates that irreversibly or quasi-irreversibly inactivate the enzyme that is responsible for their activation. This catalysis-dependent and irreversible inhibition is called mechanism-based inactivation (MBI) or suicide inactivation, because it requires at least one cycle of the CYP catalytic process, and the phenomenon is time-dependent inhibition (TDI), because the inhibition is enhanced over time. Thus, TDI is one of the main features that distinguishes reversible inhibition from MBI; mechanism-based inactivators represent a subset of time-dependent inhibitors. Practically all mechanism-based inactivators are competitive with the normal substrate of the enzyme, after which they rely on the catalytic mechanism of the enzyme active site to produce reactive intermediates (Equation 5). Compared to reversible inhibition, irreversible inhibition more frequently

results in unfavorable drug interactions as the inactivated CYP enzyme has to be replaced by newly synthesized protein (Kalgutkar et al., 2007). Thus, MBI of CYP enzymes is a major safety concern. This is because susceptibility to pharmacokinetic interactions is increased when dosed repeatedly and the duration of these interactions can be prolonged, even after removing the MBI drug. Additionally, depending on the proportion of the mechanism-based inactivator metabolized by the inactivated CYP, after multiple doses an additional clinical consequence could involve increases in systemic exposure of the inactivator itself. Finally, covalent binding of reactive metabolites to CYP enzymes can lead to hapten formation which can sometimes trigger an autoimmune response in susceptible patients (Orr et al., 2012).



Equation 5. Kinetic scheme illustrating mechanism-based inactivation (Kalgutkar et al., 2007).

Mechanism-based inactivation can be divided into irreversible and quasi-irreversible inhibition. For quasi-irreversible inhibitors, the reactive intermediates coordinate tightly to the heme iron atom which leads to the formation of a catalytically inactive metabolite-intermediate complex (MIC) with the CYP enzyme (Hines and Prough, 1980; Muakkassah et al., 1981; Correia and Ortiz de Montellano, 2005). In contrast, for irreversible inhibitors, reactive intermediates react covalently with the active site amino acid residues and/or alkylate/arylate the porphyrin of the heme, leading to destruction of heme. Sometimes the resulting heme fragments can also lead to a modification of protein (i.e., fragments that can themselves modify the protein) (Kamel and Harriman, 2013). Quasi-irreversible inhibition can be reversed in certain experimental conditions (i.e., by incubation with highly lipophilic drugs that displace the metabolic intermediate from the active site of the enzyme, by irradiation at 400-500 nm or by ferricyanide (Ullrich and Schnabel, 1973; Dickins et al., 1979)), but in physiological conditions, the blockade is irreversible and is therefore indistinguishable from the pharmacokinetic impacts of irreversible inhibition *in vivo* (Riley et al., 2007).

Multiple classes of compounds are irreversible or quasi-irreversible inhibitors. They contain several different kinds of functional groups which undergo CYP-catalyzed activation to reactive intermediates. These functional groups include: amines, epoxides, terminal acetylenes, olefins, furans and thiophenes, dichloro- and trichloroethylenes, methylenedioxyphenyls (MDPs), conjugated structures, isothiocyanates, hydrazines, thioamides and dithiocarmamates and, in general, Michael acceptors (i.e., electrophiles containing conjugated double-bond systems). The two main classes of quasi-irreversible

inhibitors are compounds with a MDP function and nitrogen compounds, usually amines (Figure 8) (Kamel and Harriman, 2013).

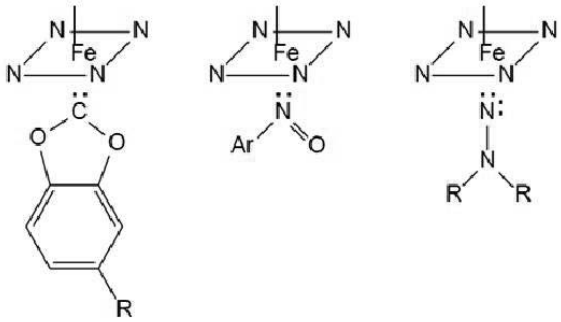


Figure 8. Proposed structures for the MIC formed during the catalytic turnover of (**left**) MDP compounds to carbene-iron complex, (**middle**) alkylamines to nitroso-iron complex and (**right**) 1,1-dialkylhydrazines to nitrene-iron complex (Lin and Lu, 1998).

The inactivation rate of CYP is proportionally dependent on the inhibitor concentration as presented in the equation below (Eq. 6):

Eq. 6

$$kobs = \frac{kinact * [I]}{KI + [I]}$$

where k_{obs} is the pseudo first-order rate constant of inhibition at the inactivator concentration [I], k_{inact} the maximum inactivation rate, and K_I the inactivator concentration when the rate of inactivation reaches half of k_{inact}.

The following specific *in vitro* criteria for mechanism-based inhibition were devised in the 1990's by Silverman (Silverman, 1995):

1) time-dependency
2) saturation
3) substrate protection
4) irreversibility
5) inactivator stoichiometry
6) catalytic step involvement, and
7) inactivation prior to release of active species.

The term MBI should be reserved for the situation when most or all of these criteria are satisfied. 1) TDI is the distinguishing feature between reversible and MBI. 2) As the concentration of inactivating compound increases, the observed rate of inactivation increases to a maximum so that a hyperbolic relationship is apparent. 3) Because the inactivating compound is a substrate, competition for binding to the same active site should occur in the presence of other substrates and competitive inhibitors (i.e., the observed rate of inactivation is decreased). 4) The inhibition of CYP should be irreversible or quasi-

irreversible so that removal of excess inactivator will not affect residual CYP activity. 5) Since reactive intermediates bind to the heme and/or the apoprotein and "block" the enzyme active site, a 1:1 stoichiometry of inactivator to CYP should be expected. This can be studied by using radioactively labelled inactivator to measure the number of inactivator molecules attached per enzyme molecule. 6) The mechanism-based inactivators are converted by CYP to reactive intermediates that inactivate the enzyme, cofactor(s) is required and catalytic turnover is essential. Thus, MBI of CYP is NADPH-dependent. 7) The inactivating species must remain within the enzyme active site prior to inactivation. If it is converted into an activated form that escapes the enzyme active site, and then returns to inactivate the enzyme, the inactivator is considered to be metabolically activated, not MBI (Polasek and Miners, 2007).

Not only clinically relevant drug interactions but also hepatotoxicity have been associated with MBI. As the primary site for drug metabolism – detoxification and bioactivation – the liver is the most important target organ for drug toxicity, and the relationships between the metabolic profile of an oral medication and adverse hepatic events have been established experimentally. Drugs that are not significantly metabolized rarely cause idiosyncratic drug-induced liver injury. Thus, MBI of CYP is also one indicator of drug-induced hepatotoxicity (Bauman et al., 2009; Gomez-Lechon et al., 2010). It appears reasonable to screen for reactive metabolite formation in early drug discovery to eliminate post-marketing failure of otherwise useful therapies (Dieckhaus et al., 2002; Pelkonen et al., 2015; Thompson et al., 2016).

2.5.3 Time-dependent inhibition (TDI)

Although testing of reversible inhibition is well established for predicting the drug interaction potential, drug developers have only recently focused on TDI. *In vitro* evaluation of drug candidates for their potential CYP inhibition has been recently implemented throughout the pharmaceutical industry. These efforts have largely led to a decline of drug development failures directly linked with CYP inhibition. Today, potential *in vivo* effects attributable to drug interactions due to competitive inhibitors can be fairly well predicted from *in vitro* CYP kinetics. Progress made with reversible CYP drug interactions has prompted a switch to study and model TDI and induction interactions. The failures of numerous late-stage clinical drug candidates have been linked with TDI, and this mechanism is also associated with liver toxicities, often encountered with preclinical candidates. The current challenge is to identify and weed out time- and concentration-dependent effects of potential time-dependent inhibitors among the vast numbers of compounds in the early drug development process (Wienkers and Heath, 2005; Fowler and Zhang, 2008; Zimmerlin et al., 2011; Yan and Caldwell, 2012; Riley and Wilson, 2015). This information is particularly important because failure to consider MBI *in vitro* can lead to a significant underestimation of the magnitude of drug interaction *in vivo*. This is especially important when trying to predict drug interactions from *in vitro* data based on competitive models (Bjornsson et al., 2003; Polasek and Miners, 2007).

Measurement of enzyme inactivation rates (k_{inact} and K_I) is technically challenging and the analysis of TDI data for CYP enzymes is not straightforward. Nonetheless, it is important to be able to determine reliably whether a drug is a TDI or not, and if so, how best to derive the inactivation kinetic parameters K_I and k_{inact}. From the point of view of

drug discovery, the characterization of the CYP inactivation kinetics is crucial for making quantitative predictions of the clinical drug interaction potential of TDI-positive drug candidates. In addition, quantitative comparison of inactivation kinetics can be used to rank compounds and establish structure-activity relationships within a series of chemicals (Orr et al., 2012; Yates et al., 2012).

The importance of reducing risks of drug interactions especially with respect to CYP TDI has been recognized currently by the regulatory agencies FDA (2012) and EMA (2012) and by the drug industry (position papers by member companies of the Pharmaceutical Research and Manufacturers of America (PhRMA) (Grimm et al., 2009). The current regulatory guidances by the EMA and the FDA for *in vitro* drug interaction studies include recommendations that time-dependent and mechanism-based inactivator properties should be tested. The PhRMA recommended the use of a tiered approach in which abbreviated assays are first used to evaluate whether or not new chemical entities demonstrate TDI, followed by more thorough inactivation studies for those that displaying that property. Given the concern around pharmacokinetic drug interactions through TDI, *in vitro* CYP TDI is routinely evaluated as a part of lead optimization. However, according to Orr et al. (2012), the identification of an *in vitro* CYP TDI liability can raise several questions:

1) What is the mechanism of TDI?
2) Does it involve the formation of reactive metabolites?
3) Is there a 1:1 correlation between CYP TDI and reactive metabolite formation (as measured from reactive metabolite trapping studies using nucleophiles)?
4) What is the likelihood that a CYP time-dependent inhibitor will also cause toxicity?
5) What are the drug interaction risk mitigation options when dealing with CYP inactivators in drug discovery: compound progression or termination?
6) Several drugs exhibit in vitro TDI of CYP enzymes, but only a fraction of them cause clinical drug interactions. Hence, when do we initiate labor-intensive medicinal chemistry efforts to design compounds devoid of CYP TDI liability?
7) What are the best methods to precisely predict the occurrence of clinical drug interactions with drug candidates that inactivate CYP enzymes?
8) What are (if any) the qualifying considerations for clinical progression of a CYP time-dependent inactivator with projected clinical drug interaction risks?

2.6 DRUG INTERACTIONS

A factor that can change the response to a drug is the simultaneous administration of other drugs and xenobiotics. Whenever two or more drugs or other xenobiotics are administered over similar or overlapping time periods, there is a possibility for drug interactions. Patients are commonly treated with more than one drug and may also be using over-the-counter medications, vitamins and food supplements, and as well as having individual dietary choices. This polypharmaceutical nature of healthcare and the ubiquitousness of self-medication requires consideration of potential drug interactions for both patients and physicians, as well as the drug industry and regulatory authorities.

There are numerous mechanisms to explain how drugs may interact, but most of them can be categorized as pharmacodynamic (antagonistic or additive effects) or pharmacokinetic (absorption, distribution, metabolism, excretion) interactions. In many

cases the interactions have a pharmacokinetic rather than a pharmacodynamics basis. Pharmacokinetic interactions lead to alterations in the plasma concentration of the object drug. A key mechanism of pharmacokinetic interactions is inhibition or induction of CYP enzymes. Further, drug interactions arising from CYP inhibition are more frequent (Cascorbi, 2012; Varma et al., 2015).

The importance of the impacts of drug interaction varies from negligible to dramatic, in the latter case, usually either through reduced efficacy or increased incidence of adverse events. Clinical consequences depend on the nature of the drug or its metabolites and the magnitude of the change in the concentration of active compounds at the site of pharmacological action. Drugs most likely to pose interaction problems are those having a narrow therapeutic index (i.e., small difference between therapeutic dose and toxic dose), extensive first-pass metabolism (i.e., the loss of drug as it passes through the liver for the first time), steep dose–response curve or a single, inhibitable route of elimination (Byrne, 2003). In addition to adverse effects, even deaths, drug interactions have resulted in early termination of drug development, refusal to be granted a market licence, severe prescribing restrictions and withdrawal of drugs from the market (Backman et al., 2002; Kalgutkar et al., 2007; Pelkonen et al., 2008; Onakpoya et al., 2015).

When a drug interaction occurs, the drug causing changes in CYP activity is referred to as the "perpetrator" of the drug interaction, whereas the coadministered drug that is affected by the change in CYP activity is referred to as the "victim" or "object" of drug interaction. Usually, a perpetrator is a potent inhibitor or inducer of drug-metabolizing enzymes and/or drug transporters, whereas a victim is a substrate of that enzyme or transporter (Bode, 2010; Orr et al., 2012).

Publications about unexpected drug interactions began to appear in the literature in the 1970s. Throughout the 1980s, there were increasing numbers of clinical case reports of drug interactions involving various drugs. For example, in 1978, a serious drug interaction was reported between digoxin and quinidine, in which digoxin levels more than doubled in patients concomitantly receiving quinidine. In 1990, it was reported that the interaction between terfenadine and ketoconazole caused sudden death, which together with other reports of adverse effects, contributed to the removal of terfenadine and several other drugs, including astemizole, cerivastatin, cisapride and mibefradil from the market (Huang et al., 2008). The drug withdrawals due to interactions emphasized the need for a systematic preapproval evaluation of potential drug interactions. During the last 30–40 years the general problem of pharmacokinetic drug interactions has received increasing attention. Foreseeing clinically significant drug interactions during drug development is currently a major challenge for the pharmaceutical companies and regulatory agencies.

Computer-based programs have become available for screening potential adverse drug interactions during drug prescribing and dispensing (Tamblyn et al., 2003; Heikkila et al., 2006; Bottiger et al., 2009; Toivo et al., 2016). The University Pharmacy (owned by the University of Helsinki) and two other community pharmacies began first to use a prospective drug interaction surveillance system [based on the FASS (Farmaseutiska Specialiteten i Sverige) database] in 2004. The surveillance system of that time was linked with the prescription processing software (Linnea®). While a prescription was processed, the surveillance system checked automatically for possible interactions among the client's drugs in the prior 13–24 months prescriptions from any University Pharmacy outlets or the particular private pharmacy. This new service model demonstrated that community

pharmacies could actively contribute to the management of the drug interaction risk and systematically use their surveillance systems for identifying patients at risk of experiencing clinically significant drug interactions (Heikkila et al., 2006; Toivo et al., 2016). Furthermore, physicians' use of such databases has been shown to reduce the risk of serious drug interactions in primary health care (Andersson et al., 2013).

The Swedish Finnish Interaction X-referencing drug interaction database (SFINX-PHARAO, today INXBASE) is a commonly used commercial drug interaction database and software providing brief and concise evidence based information concerning the consequences of and recommendations for over 20000 drug combinations. It has been available since 2005 and in Finland it is the main database and clinical decision tool that delivers information about potential drug interactions and their clinical relevance at the time of drug prescription and drug dispensing process. The database is updated four times a year by Medbase Ltd in Turku, Finland, the Karolinska Institute Department of Clinical Pharmacology in Stockholm and the Stockholm County Council, Sweden. Interactions are classified according to their clinical significance (A–D) and level of documentation (0–4). Level A indicates a clinically insignificant interaction and D a clinically significant interaction that should be avoided. Level 0 indicates that data is derived from extrapolation on the basis of studies with similar drugs and 4 that data is derived from controlled studies in relevant patient populations. Thus level D4 indicates the highest level of evidence (Bottiger et al., 2009).

Currently, electronic prescriptions (ePrescriptions) have improved medication safety in many areas, including management of patients' overall medication because prescriptions are visible in the Prescription Centre, thus making it easier to detect drug-related problems, such as drug interactions (Kauppinen et al., 2017).

Although the CYP2C19 enzyme catalyzes the metabolism of fewer drug substrates than some other CYPs, for example CYP3A4 and CYP2D6 enzymes, it plays an important role in the metabolism of most proton pump inhibitors (PPIs), e.g. omeprazole; antidepressants, e.g., citalopram and amitriptyline; antiepileptics, e.g., mephenytoin; antiplatelet drugs, e.g., clopidogrel and the anxiolytic drug, diazepam. CYP2C19 is also partially responsible for the metabolism of several other drugs, such as the antipsychotic clozapine and the β-blocker propranolol (Lee, 2012). Concurrent use with other CYP2C19 substrates or inhibitors can have unexpected consequences due to changes in plasma concentrations.

For example, combination therapy with PPIs is often recommended to attenuate gastrointestinal bleeding risk, particularly during dual antiplatelet therapy with clopidogrel and acetylsalicylic acid. Combination of clopidogrel and omeprazole/esomeprazole is designated as a class C4 interaction in the INXBASE database, indicating a reduction in clinical efficacy of clopidogrel. It has been observed that drug interactions do not represent a class effect for PPIs, which means that each individual PPI can induce unique drug interactions. Concomitant use of those PPIs without strong affinity to CYP2C19 with clopidogrel could be justified in patients with a high risk of bleeding (e.g. patients with prior upper gastrointestinal bleeding, Helicobacter pylori infection, advanced age, steroid treatment and nonsteroidal anti-inflammatory drug use) (Yucel et al., 2016). Lansoprazole, pantoprazole and rabeprazole are designated as a B4 class of interaction in combination with clopidogrel, being thus better choices among the PPIs (Scott et al., 2014).

The benzylisoquinoline alkaloid noscapine, a popular antitussive drug, is associated with clinically significant drug interactions (class D2) when taken with warfarin (Ohlsson et

al., 2008; Scordo et al., 2008; Fang et al., 2010). Noscapine has been shown to inhibit CYP2C9 and CYP3A4 *in vitro* in a time-dependent manner (Fang et al., 2010). Noscapine caused clear inhibition of CYP2C9 and CYP2C19 activity in an *in vivo* study (Rosenborg et al., 2010).

The benzylisoquinoline alkaloid drug papaverine, the vasodilator (muscle relaxant), is associated with clinically significant drug interactions (class D0) with 40 drugs causing most often prolongation of QT-interval and increasing risk for torsades de pointes (Morissette et al., 2005; Wible et al., 2005).

2.6.1 Herb-drug interactions

As described in section 2.1.4 (Traditional medicines), NPs and HMPs are widely and increasingly used all around the world. This important group of multicomponent therapeutics (see Figure 4, page 14) contains usually more than one active compound, in fact, the active ingredients are frequently unknown. Even the desired pharmacological effects cannot be attributed to one active ingredient in traditional herbal medicinal products, but rather to integrated effects resulting from the combined actions of multiple ingredients. In addition, NPs and HMPs are often administered in combination with conventional drugs. In recent years, the issue of herbal product-drug interactions (i.e., herb-drug interactions) has generated increasing concern. Such interactions can increase the risk for an individual patient, especially regarding drugs with a narrow therapeutic index (such as warfarin, ciclosporin and digoxin). Although herbal remedies are perceived as being natural and therefore safe or regarded as low risk because of their natural origin and their long history of human use, many have adverse effects that can sometimes produce life-threatening consequences (Di Lorenzo et al., 2015). In addition, both potential and clinical herb-drug interactions have been reported in a wide variety of laboratory, animal and human studies and case reports (He et al., 2011; Shi and Klotz, 2012; Wu et al., 2012). The majority of the interactions may be difficult to predict and sometimes the results are contradictory. The clinical implications of herb-drug interactions depend on a variety of factors, for example, the co-administered drugs, patient characteristics, origin of the NPs, composition of the NP's constituents and the applied dosage regimens (Shi and Klotz, 2012).

Because NPs represent a mixture of organic compounds, they are eliminated by the same system of xenobiotic metabolism and transport that clear also synthetic drugs from the body. Thus, from a mechanistic perspective, pharmacokinetic herb-drug interactions appear inevitable and comparable with those recognized drug-drug interactions. However, it is difficult to conduct any evaluation of the actual quantity and importance of herb-drug interactions prevalence, because herbal preparations, as a rule, do not require any pre-market safety and efficacy testing nor any post-marketing surveillance (Hermann and von Richter, 2012).

Pharmacokinetic studies involving absorption, distribution, metabolism and excretion (ADME) are today integrated into drug development, but ADME studies are generally not needed prior to marketing of herbal products. Thus, for the majority of herbal remedies, data on their ADME properties in humans are often lacking. Because the same basic (natural) laws are valid no matter the origin of a compound(s) (e.g., natural or man-made),

in this regard, the safe and optimal use of natural products would require a full understanding of their ADME profiles. However, even though there has been an increasing number of ADME trials of herbal remedies, these studies have mainly focused on a small number of the most commonly used herbal medicinal preparations. Many reviews provide evidence of potential pharmacokinetic and/or pharmacodynamic interactions involving about six to 11 common herbal medicinal products, which are all widely used in the Western world (Finnish names in parentheses): St John's Wort (mäkikuisma), black cohosh (tähkäkimikki), echinacea (punahattu), garlic (valkosipuli), goldenseal, ginkgo (neidonhiuspuu), kava, milk thistle (maarianohdake), panax ginseng (ginseng), panax quinquefolius (amerikanginseng) and saw palmetto (sahapalmu) (Shi and Klotz, 2012; Wu et al., 2012).

In addition, there are a large number of reports in the literature of various extracts and constituents of NPs as potent inhibitors of transporters and drug metabolizing enzymes. However, without standard methods for NP characterization or *in vitro* testing, it is difficult to extrapolate these reports to clinically relevant herb-drug interactions. This **lack of a clear definition of risk prevents clinicians and consumers from making informed decisions about the risks associated with taking NPs with conventional medications** (Roe et al., 2016). Probably the best known is the interaction potential of St John's wort extracts which can result in significant induction of CYP3A4. The magnitude of CYP3A4 induction is correlated significantly with the hyperforin content in the St John's wort extracts. In addition, other individual constituents of St John's wort have different inhibitory effects on CYP enzymes; St John's wort extracts can inhibit CYP3A4, CYP2C9, CYP1A2, CYP2D6, CYP2C19 and CYP1B1 (Shi and Klotz, 2012).

Goldenseal is known to contain various isoquinoline alkaloids, such as berberine, hydrastine, berberastine, hydrastinine, tetrahydroberberastine, canadine and canalidine (of which berberine and hydrastine are the main active constituents). Goldenseal root extract have been standardized to contain 24.1 mg isoquinoline alkaloids per capsule and is reported to inhibit CYP2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 enzymes (Hermann and von Richter, 2012; Shi and Klotz, 2012; the International Agency for Research on Cancer (IARC) monograph volume 108 (2016) available at Internet: http://monographs.iarc.fr/ENG/Monographs/vol108/index.php). This means that goldenseal has the potential to cause metabolism-based drug interactions when coadministered with drugs that are metabolized via these enzymes.

In addition to the INXBASE, Medbase Ltd (in Turku) maintains also the Herbalbase database that provides evidence based analysis on the efficacy and safety of herbal medicines. In this software, interactions between drugs and herbal medicines have been described and they are classified similarly as in the INXBASE database (A–D). For example, the herbs that have exhibited the greatest potential (class D) to interact with warfarin include (Finnish names in parentheses) berberine (berberiini), goji (gojimarja), milk thistle (maarianohdake), St. John's wort (mäkikuisma), willow bark (paju), Melilotus officinalis (L.) Lam. herba (rohtomesikkä) and saw palmetto (sahapalmu). Leite et al. (2016) lists also garlic (valkosipuli), ginger (inkivääri), ginkgo biloba (neidonhiuspuu) and ginseng (ginsengjuuri). In addition, according to the Herbalbase database, altogether 15 herbal medicines in class C potentially interact with warfarin. Of these, garlic and gingko are the most frequently used among community-dwelling elderly individuals and both of these

supplements (along with some others mentioned above) have the potential to interact with anticoagulants and produce bruising or bleeding problems (de Souza Silva et al., 2014).

It is clear that possible harmful interactions between multi-ingredient natural products and drugs is a topic of paramount importance. If we are to optimize the use of herbal remedies, it is clear that further studies and new methods to explore their ADME properties in humans are undoubtedly necessary. From this perspective, the current *in vitro* approach designed for single drugs may be a feasible starting point (Sevior et al., 2010).

2.6.2 Guidelines for the investigation of drug interactions

The evaluation of interaction potential of an investigational drug is an integral part of the risk assessment to be conducted during drug development and review by regulators. Drug interaction studies explore whether a drug candidate affects the pharmacokinetics of marketed drugs and conversely, whether marketed drugs affect the pharmacokinetics of the drug candidate. Drug candidates are thus evaluated for their ability to induce or inhibit CYP enzymes or P-glycoprotein (P-gp) or to find out if drugs are substrates for CYPs or P-gp or any other transporter.

During the period of 1987 to 1991, mainly *in vivo* studies were used with likely coadministered drugs, whereas between 1992 and 1997, the majority of studies involved *in vitro* procedures and metabolic mechanisms. Regardless of some restrictions in the extrapolation of *in vitro* drug metabolism data to the *in vivo* situation, *in vitro* studies have remained important initial evaluations since 1997 in this area principally because of the reduced cost compared with *in vivo* studies and the high throughput nature of *in vitro* examinations (Alfaro, 2001).

The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have issued guidances for industry on drug interaction studies. FDA published its first guidance on drug interactions in 1997, followed by a second guidance in 1999, a third in 2006 and the most recent one in February 2011. The EMA also published guidances in 1995 and 2010, with the latest draft was issued in 2012. These outline comprehensive recommendations on a wide variety of *in vivo* and *in vitro* studies to assess potential drug interactions; usually through *in vitro* studies followed by *in vivo* studies (EMA, 2012; FDA, 2012; Prueksaritanont et al., 2013). These guidances include best practices for *in vivo* and *in vitro* studies, together with study design, dosing, statistical analysis and guidance for appropriate labeling. *In vitro* and *in vivo* studies are conducted mainly for metabolism and transporter-based drug interactions. Research in the drug industry has focused on screening drug candidates for possible drug interaction liability based on the limited *in vitro-in vivo* correlation models currently available. Models that can precisely predict clinical outcomes are presently lacking.

These guidelines state that pharmacokinetic interaction studies should generally be carried out in humans or with human enzymes and transporters. *In vivo* interaction studies should be performed in healthy adults and studies usually have a cross-over or sequential design. The *in vitro* studies should be performed before phase I clinical studies start and those enzymes contributing to ≥ 25% of the oral clearance should be confirmed if possible *in vivo* (EMA, 2010; Prueksaritanont et al., 2013). EMA and FDA have recommendations about which enzymes should be tested in inhibition studies. The following CYP enzymes are

recommended for routine assessment: CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Zhang et al., 2010).

In vitro inhibition can be studied by using hepatocytes or other cells expressing the investigated enzyme or human liver microsomes. The inhibition mechanisms (e.g. reversible or TDI) and inhibition potency (e.g. K_i) are generally studied using a marker substrate to measure the enzyme activity under linear substrate metabolism conditions. The effect of a wide range of concentrations of investigational drug are investigated and the inhibitory constant (K_i) is determined. Known strong inhibitors should be used as positive controls in the studies and their K_i should be determined and compared to reference values. These studies should also evaluate whether pre-incubation with the investigational drug changes the inhibitory potency of the drug. If the pre-incubation has an effect on the inhibitory potency, more detailed studies are needed. If the inhibition is enhanced by pre-incubations, TDI is present. For mechanism-based inactivators, k_{inact} and K_I should be determined. In addition, it is recommended to investigate the enzyme inhibitory effect of major metabolites. Examples of probe substrates and inhibitors for *in vitro* studies are listed in Table 4 (FDA, 2006; EMA, 2012).

Table 4. Examples of well validated inhibitors, substrates and marker reactions of specific enzyme activities *in vitro* (FDA, 2006; EMA, 2012).

Enzyme	**Inhibitor**	**Substrate and marker reaction**
CYP1A2	furafylline[1][2]	phenacetin O-deethylation[1][2]
CYP2A6	tranylcypromine[2], methoxsalen[2]	coumarin 7-hydroxylation[2], nicotine C-oxidation[2]
CYP2B6	ticlopidine[1][2], thiotepa[1], clopidogrel[2]	efavirenz hydroxylation[1][2], bupropion hydroxylation[1][2]
CYP2C8	montelukast[1][2], quercetin[2]	paclitaxel 6-hydroxylation[1], amodiaquine N-deethylation[1], taxol 6-hydroxylation[2]
CYP2C9	sulfaphenazole[1][2]	S-warfarin 7-hydroxylation[1][2], diclofenac 4'-hydroxylation[1][2], tolbutamide methyl-hydroxylation[2]
CYP2C19*	ticlopidine[1][2], nootkatone[1][2], -(-)-N-3-benzyl-phenobarbital[1], loratadine[1]	S-mephenytoin 4'-hydroxylation[1][2]
CYP2D6	quinidine[1][2]	bufuralol 1'-hydroxylation[1][2], dextromethorphan O-demethylation[2]
CYP2E1	diethyldithiocarbamate[2], clomethiazole[2], diallyldisulfide[2]	chlorzoxazone 6-hydroxylation[2]
CYP3A(4)#	ketoconazole[1][2], itraconazole[1][2]	midazolam 1'-hydroxylation[1][2], testosterone 6β-hydroxylation[1][2]

*presently no specific inhibitor known for *in vitro* use. Listed inhibitor(s) are not specific but can be used together with other information or in a single enzyme system.
#CYP3A inhibition should be investigated using both marker reactions, i.e., two structurally unrelated substrates should be evaluated.
[1] EMA; [2] FDA

To minimize unwanted clinical consequences, it is crucial to understand the mechanism of drug interactions and manage the interplay of various factors affecting these interactions. The drug interaction field has enjoyed significant advances, driven by nonclinical and clinical experience, technological advances, improved understanding of the molecular basis of drug interactions, and a continuous dialogue between the regulatory authorities and scientists in pharmaceutical industry (Bode, 2010).

The evaluation of potential interactions is recommended now also for new herbal preparations and this should be investigated by the applicant (EMA, 2010). The interaction potential of one particular HMP or food product is difficult to extrapolate to other preparations produced from the same raw source material. Typically, the interacting compounds have not been sufficiently identified and therefore analysis of the product constituents may not be used to extrapolate towards the magnitude of the interaction effect. For traditional and well-established herbal products, the potential for interaction should be elucidated if there are indications of clinically significant interactions in humans. Usually there is no data on the pharmacokinetics of the ingredients of herbal preparations or herbal substances and therefore the *in vivo* relevance of *in vitro* data cannot be evaluated. However, if *in vivo* data suggests that ingredients of the herbal preparation or the herbal preparation itself may cause clinically relevant drug interactions, *in vitro* studies on the enzyme inhibitory potential are recommended as such studies may reveal a causal relationship (EMA, 2010).

2.7 METHODS FOR STUDYING CYP INHIBITION IN VITRO

In the past 20 years, various fast, reliable and cost-effective *in vitro* CYP inhibition assays have been developed to evaluate potential inhibition of human CYP enzymes by pharmaceutical compounds. These assays are used at different stages of drug discovery and development in the pharmaceutical industry (Figure 9) (Obach et al., 2005; Pelkonen and Raunio, 2005; Lahoz et al., 2008; Yao et al., 2012). 1) Recommendations from several regulatory guidelines and different workshops; 2) increasing knowledge about CYP enzymology; 3) the availability of improved analytical technology and reagents, such as expressed human CYP enzymes and probe substrates; and 4) selective chemical CYP inhibitors have allowed the pharmaceutical industry to study CYP inhibitory activity at early stages of drug discovery and development (Obach et al., 2006). Data from these assays are primarily used to select clinical candidates, to design clinical drug interaction studies and for regulatory submissions for new chemical entities and drug applications (Obach et al., 2005). In general, the ability to use *in vitro* human CYP data for *in vivo* predictions is the main route for generating the data.

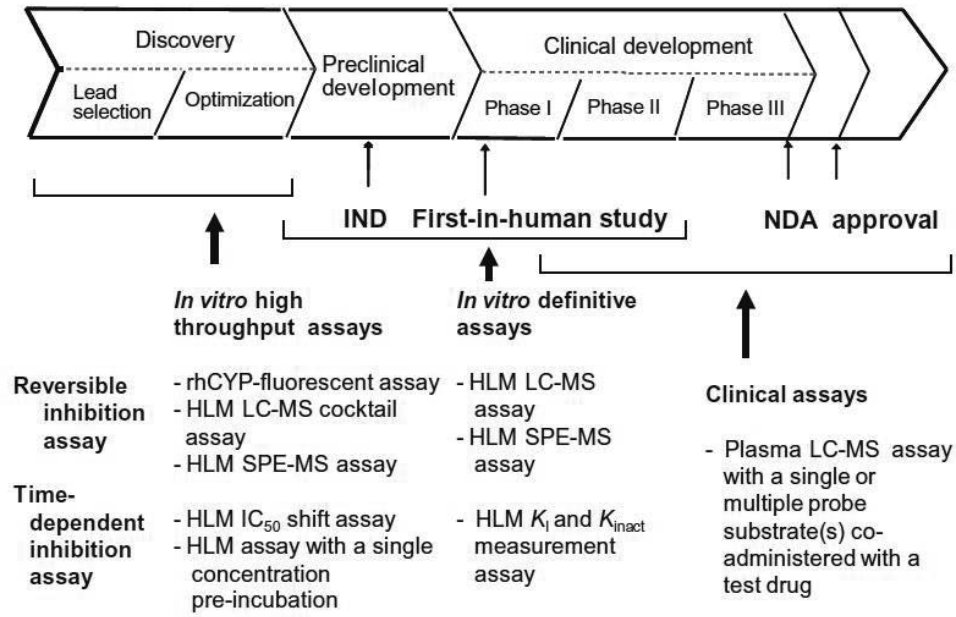


Figure 9. Assessment of CYP inhibition in drug discovery and development; **IND**, investigational new drug; **NDA**, new drug application (Yao et al., 2012).

During the past 20 years, regulatory requirements have been issued for the selection and performance of CYP inhibition assays, and all reagents for CYP inhibition assays are available (see e.g. Table 4, page 42). Additionally, some *in vitro* CYP inhibition data originating from several assays have been verified in clinical drug interaction studies. Accordingly, analytical methodologies and strategies for evaluation of *in vitro* CYP inhibition are maturing (Walsky and Obach, 2004; Fowler and Zhang, 2008; Grimm et al., 2009).

Table 5 summarizes the current applications of common *in vitro* CYP inhibition assays and their working principles, advantages and limitations. Fluorescent assays with recombinant human CYP (rhCYP) enzymes were the first types to be widely used to assess CYP inhibition in lead optimization stage in a high-throughput manner. Human liver microsomes (HLM) assays designed for luminescence or radioactive probe substrates provide reliable CYP inhibitory data also in a high throughput fashion. Unfortunately, because these probe substrates are not available for all of the major CYP enzymes and due to other limitations, these assays are no longer employed in most drug metabolism laboratories. The HLM assays with accepted probe substrates and mass spectrometry (MS), have become the methods of choice for evaluating *in vitro* inhibitory potency and are used for both reversible and time-dependent CYP inhibition. These assays are based on an HLM liquid chromatography-mass spectrometry (LC-MS) assay with a single probe substrate, HLM cocktail assays, and HLM (online) solid-phase extraction (SPE) and MS without an LC column (HLM SPE-MS) assays with a single substrate (Table 5) (Yao et al., 2012).

Microtiter plate assays are conducted using a nonspecific fluorescent substrate and a single rhCYP enzyme. They are characterized by their high throughput capability, low operation cost and ease of operatione. After or during 10–60-min incubations e.g. in 96- or 384-well plates, results are quickly recorded using a plate reader. Thus, fluorescent assays

have been commonly used for a large number of compounds in lead selection and optimization, that is called a high-throughput screening (HTS) (Moody et al., 1999; Bapiro et al., 2001; Stresser et al., 2002; Cohen et al., 2003; Donato et al., 2004; Di et al., 2007). Currently, the rhCYP-fluorescence assays are primarily used to rank discovery compounds. They are especially applicable to determine relationships between structure and CYP inhibitory activity. These assays allow for the termination of further development of NCEs that are potent inhibitors of major human CYP enzymes. The assays also support the design of NCEs with better CYP inhibition profiles. However, because most pro-fluorescent probe substrates are not CYP enzyme-selective, these assays cannot be used with HLMs. Furthermore, rhCYP-fluorescent assays are also not suitable for fluorescent test compounds. Therefore, HLM high-throughput assays, including the HLM assay with multiple probes and HLM MS assays, have replaced rhCYP-fluorescent assays in lead optimization. Thus, CYP inhibition assays using HLM and CYP probe substrates are often carried out during the later stages of drug development (Figure 9 and Table 5) (Yao et al., 2012).

Table 5. Comparison of common *in vitro* CYP inhibition assays (Yao et al., 2012).

Assay	**Advantages**	**Limitations**	**Current Applications**
Fluorescent	High throughput Low cost Easy to operate	Not suited for HLM rhCYP may yield false-positive data and is not suited for regulatory filing Not applicable to fluorescent compounds	Ranking of numerous compounds with the same chemotypes Used when HLM availability or cost is an issue
Radiometric	Fast Suited for HLM	Radiolabeled probe substrate needed Specific facilities and personnel needed to handle radioactive materials	Not commonly used
Luminescence	High throughput Easy to operate Suited for HLM	Specifically designed probe substrates needed Not available for most CYP enzymes	Not commonly used
LC-MS with a single substrate	Suited for HLM Applicable to various compounds Suited for TDI analysis Suited for regulatory filing	Requires an LC-MS instrument and operator Medium throughput Costs relatively more than a cocktail assay	Commonly used in late discovery and development
LC-MS with multiple substrates (cocktail assay)	High throughput Relatively low cost Suited for HLM	LC-MS instrument and operator needed May not be suited for regulatory purposes Not suited for TDI analysis	Used in the early discovery stage for fast and low-cost screening
MS without LC	High throughput Suited for HLM Suited for regulatory filing Suited for TDI analysis	Requires an expensive MS instrument Costs more than a cocktail assay	Widely applied from early discovery to development

2.8 IN VITRO/IN VIVO EXTRAPOLATION

The concept of intrinsic clearance (CL_{int}) is very valuable in the extrapolation of *in vitro* kinetic data to metabolic activity *in vivo*. Actually, CL_{int} can be considered as representing the basis for extrapolation of metabolic interactions from *in vitro* to *in vivo*. CL_{int} is defined as the enzymatic reaction velocity (v) divided by substrate concentration [S]. The CL_{int} is a measure of the drug metabolising activity (V_{max}/K_m) in the liver. The V_{max} value of a drug substrate can be decreased or its K_m value increased, depending on the underlying mechanism of the inhibitor. Therefore, irrespective of the mechanism, enzyme inhibition always causes a decrease in the CL_{int} and conversely, enzyme induction invariably results in an increase in CL_{int}, as a result of increased V_{max}. In the absence of inhibitor, the CL_{int} can be described by equation 7 (Lin and Lu, 1998; Lin and Lu, 2001):

Eq. 7

$$CLint = \frac{Vmax}{Km + [S]}$$

The extent of a drug interaction due to inhibition of metabolic clearance can be predicted, in theory, using the ratio of concentration of the inhibitor ([I]) to the inhibition constant (K_i). Thus, the assessment of the inhibition potential for reversible inhibitors is based on the [I]/K_i ratio. A value of < 0.1 usually indicates a low risk of a drug interaction and a value of > 1 indicates a high risk (Tucker et al., 2001; Ito et al., 2004). Biochemical principles note that when the drug (i.e., substrate) metabolism is reversibly inhibited by a second drug (i.e., an inhibitor), the CL_{int} of substrate is reduced by a factor related to the inhibitor concentration available to the enzyme [I] and the inhibition constant, K_i (Eq. 8). Normally it is not possible to measure the inhibitor concentration available to the hepatic enzyme *in vivo* in humans. Predictions have been attempted by using different inhibitor concentrations, i.e., the plasma total or unbound concentration or hepatic input concentration of the inhibitor (Ito et al., 2004). EMA (2012) recommends to use the unbound mean Cmax obtained during treatment with the highest recommended dose and FDA (2012) the maximal total (free and bound) systemic inhibitor concentration in plasma. The difference between competitive and uncompetitive (or mixed) inhibition mechanisms is not relevant when the substrate concentration is much lower than the K_m value, which is the usual *in vivo* situation that results in linear kinetics (Ito et al., 2004).

Eq. 8

$$CLintI = \frac{CLint}{1 + \frac{[I]}{Ki}}$$

where $CLintI$ signifies the value when the inhibitor is present. This theory, and the usefulness of equation 8 to describe *in vivo* data for reversible inhibition, has been confirmed in numerous animal studies. These studies have been carried out in well defined steady state conditions for various levels of inhibition, such as, in the decrease in clearance of diazepam caused by omeprazole (Zomorodi and Houston, 1995), of theophylline by

enoxacin and ciprofloxacin (Davis et al., 1994) and of antipyrine by fluconazole and ketoconazole (Ervine et al., 1996).

Although it is straightforward to measure metabolic drug interactions *in vitro*, the correct extrapolation and interpretation of *in vitro* interaction data to *in vivo* situations need to be based on a good understanding of pharmacokinetic principles. In addition, several significant factors should be taken into account, when the clinical importance of drug interactions is predicted (Lin and Lu, 1998). For example, these inhibitor related aspects are *in vitro* kinetic constants of the inhibitor, inhibitor concentration used in the predictions, changes of inhibitor concentration over time, active transport, parallel interaction mechanisms, inhibitory/inductive metabolites and protein binding. Correspondingly, substrate related aspects are substrate f_m (i.e., the fraction metabolized by the inhibited enzyme), parallel pathways of metabolism, protein binding, extraction ratio and therapeutic index. Finally, the clinical significance is dependent on the extent of the change in the concentration of active compounds at the site of pharmacological activity (parent drug and/or active metabolites). The therapeutic index of the drug is also important; the smaller the difference between effective and toxic concentration, the greater the probability that a drug interaction has severe clinical consequences (Lin and Lu, 1998; Cascorbi, 2012). In fact, there are examples of successful predictions of *in vivo* metabolic interactions based on the studies of metabolic processes *in vitro* (Olkkola et al., 1994) as well as in drug interactions where their magnitude and mechanisms would have been difficult or impossible to predict with available *in vitro* tools (Bode, 2010).

TDI of CYP enzymes by the drug candidate may confuse the prediction of the magnitude of drug interactions *in vivo*. For example, drug interactions predicted from *in vitro* data, based upon competitive models, may underestimate the real interaction that could occur *in vivo* in the case of a time-dependent inhibitor (Bjornsson et al., 2003). The prediction of the clinical importance of mechanism-based inactivation based on *in vitro* studies is more complicated than that of reversible inhibition. *In vitro* kinetic evaluation and prediction of drug interaction produced via reversible inhibition and mechanism-based inactivation rely on operationally and conceptually different approaches. Drug interaction risk assessment requires an estimation of inactivator potency (K_I) and maximal inactivation rate (k_{inact}) *in vitro*. In order to predict the magnitude of the drug interaction, both need to be considered in the context of the biological turnover rate of the enzyme (k_{deg}) and clinical exposures of the inactivator (I), respectively. Since inactivation is both concentration- and time-dependent, risk assessment cannot be conducted by a simple comparison of inactivator potency against *in vivo* exposure. As well as the [I]/K_I ratio, the k_{inact}/k_{deg} ratio is an independent factor in determining the inhibition potential. Therefore, even if the [I]/K_I ratio is very small, the clinical interaction can be relevant in the case of very rapid inactivation, making the k_{inact}/k_{deg} ratio sufficiently large. MBI contour plots tracking combinations of [I]/K_I and k_{inact}/k_{deg} resulting in identical fold-reductions in CL_{int} are proposed as a valuable framework for drug interaction risk assessment (Venkatakrishnan and Obach, 2007).

In the extrapolation of the *in vivo* potency of a mechanism-based inactivator, the following equation (Eq. 9) can be used:

Eq. 9

$$\frac{kinact * [I]}{kdeg} * (KI + [I])$$

If the obtained index is << 1, a weak inhibition can be expected, with a victim drug AUC fold increase of ≤ 2 in clinical study subjects. The opposite relates to moderate and potent inhibition (Zhou and Zhou, 2009). The clinical impact of mechanism-based inhibition on CYP mediated clearance can be predicted from *in vitro* inactivation data using several mathematical models (Mayhew et al., 2000; Yamano et al., 2001). These mathematical models are generally based on a number of simplifying assumptions (Lu et al., 2003).

2.9 MOLECULAR MODELING (IN SILICO) OF CYP ENZYMES

Molecular modeling originates from the need to describe molecular phenomena invisible to the human eye in order to be able to understand and predict molecular structures, physical and chemical characteristics and behavior (Höltje et al., 2003). Especially during the last 30 years, the increased sophistication of molecular modeling has been closely associated with the development of computers, or rather the modeling methods have been developed in terms of computer technology and currently they are understandably inseparable. Proteins, for example, would not have been measurable with methods such as X-ray structure analysis and nuclear magnetic resonance without the corresponding computer technology. A great variety of molecular modeling approaches have been applied to CYP enzymes (described in Figure 10), successfully adding to our understanding of CYP structure and function in a way that is complementary to experimental studies (de Graaf et al., 2005).

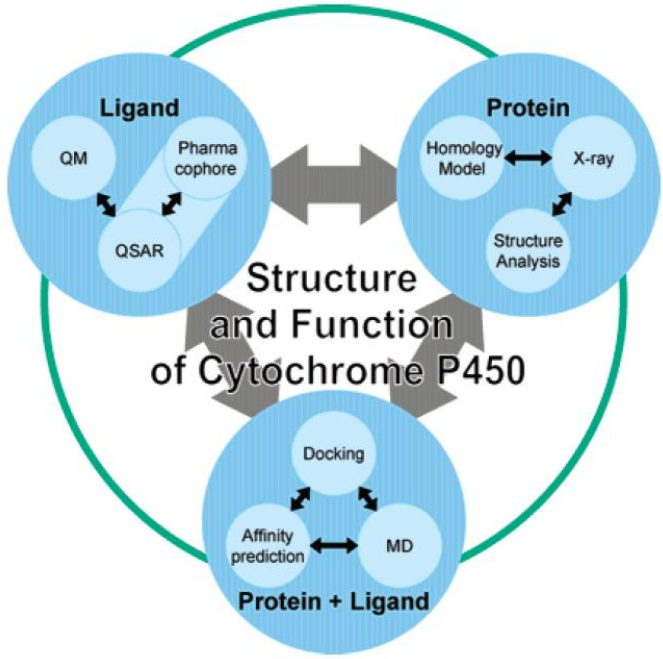


Figure 10. Schematic arrangement of CYP modeling methods and the division into ligand-based, protein-based and combined methods. Arrows indicate relationships among methods and method groups. Ligand-based methods are quantum mechanical (QM) calculations and classical and QSAR pharmacophore models. Protein-based methods are protein homology, X-ray crystallography, model building and protein structure analysis. Combined methods are molecular dynamics (MD) simulations, automated ligand docking, and affinity prediction calculations (de Graaf et al. 2005).

In computer-aided drug design, inter alia, the quantitative structure-activity relationship (QSAR) analysis and molecular docking are useful tools. In this thesis, the focus will be on those methods as they have been widely used for CYPs. There always exists a relationship between the physico-chemical characteristics of the drug molecule and its biological activity. Three-dimensional (3D) QSAR approaches use statistical methods (chemometrical methods) to correlate this variation in biological or chemical activity with information on the three-dimensional structure for a compound. For example, 3D-QSAR methods include CoMFA (Comparative Molecular Field Analysis) (Cramer et al., 1988), CoMSIA (the Comparative Molecular Similarity Indices Analysis) (Klebe et al., 1994) and GRID/GOLPE (Goodford, 1985; Baroni et al., 1993), the results of which can be represented by three-dimensional feature maps, in which the biological properties are represented by the steric and electrostatic fields around the molecule.

The basic concept is that the steric and electrostatic fields can provide all the necessary information to understand the biological properties for a set of compounds. Different fields of molecules can thus be compared with each other and the differences between biological activities of the molecules should be reflected in the field maps. Based on these field maps, a model can be created in which the maps are used to calculate the biological activity of the molecule (Höltje et al., 2003). Some examples of 3D-QSAR models for CYP enzymes: 1A2 (Korhonen et al., 2005), 2B6 (Korhonen et al., 2007), 2D6 (Mo et al., 2012), CYP19 (aromatase) (Oprea and Garcia, 1996), 2A6 (Poso et al., 2001; Rahnasto et al., 2005; Rahnasto et al., 2008), 2C9 (Afzelius et al., 2001; Afzelius et al., 2002), and 3A4 (Shityakov et al., 2014). The results and conclusions from QSAR analyses are providing important insights into the nature of the compounds that can act as substrates and inhibitors of the individual CYP enzymes reviewed by Sridhar et al. (2012) and Raunio et al. (2015).

When the crystal structure (X-ray structure) of the target enzyme is available, it can be used for docking molecules into the active site of the enzyme in order to determine and predict the binding. If there is no experimental 3D structure available, it is possible to generate a homology model for the protein. Therefore, molecular docking can be used to study the interaction between protein and ligands at the binding site. In addition, docking can be used for determining the oxidation potentials of sites on the molecule to predict metabolism. Docking consists of two parts. First, for the prediction of the ligand conformation and its position and orientation within sites (called the pose), the ligand is docked into the protein binding site. This is the actual docking process and involves sampling the conformational space of the ligand within the protein binding site. In the second step, the generated docking poses are evaluated and some measure of binding affinity or the fit of the ligand for the enzyme is evaluated. This step is also referred to as scoring (Kirchmair et al., 2012). The ligand will potentially bind to the protein if the geometry of the ligand-protein pair is complimentary and involves favourable biochemical interactions. With CYP enzymes, the most common docking methods applied include GOLD, FlexX, DOCK and AutoDock reviewed by de Graaf (2005) and Olsen et al. (2015). Some examples of docking studies for CYP enzymes: 1A2 (Vasanthanathan et al., 2009), 1A2 and 3A4 (McEneny-King et al., 2017), 1B1 (Kumar and Gupta, 2016; Poirier et al., 2016), 2D6 (Unwalla et al., 2010), 3A4 (Shityakov et al., 2014), and 2C9, 2D6 and 3A4 (Ramesh and Bharatam, 2014).

Models created in aid of molecular modeling can be used to examine a number of biologically untested molecules and try to predict their properties, e.g., are they

metabolized by means of a specific enzyme and do they inhibit its activity when they may cause potential adverse drug interactions. Predictive models can be used in the early stages of the drug development process to screen out unfavorable molecules or start the appropriate modifications to their structures in order to optimize the properties of the compounds. However, the underlying experimental data behind the models must be of good quality, because it always represents the basis of the hypothetical model. Each model can test similar properties of unknown compounds, which have been taken into account in the creation of the model with biologically tested molecules (White, 2000; Höltje et al., 2003; de Graaf et al., 2005). In addition, Mao et al. (2006) showed (by using the QSAR modeling of CYP3A4 enzyme inhibition with four large data sets of *in vitro* data) that the traditional QSAR model applied to one data set does not lead to predictive models that would be useful for *in silico* filtering of chemical libraries and presents a multiple pharmacophore hypothesis that is a conceptual extension of the conventional QSAR approach. However, CYP3A4 is evidently the most challenging of the CYP enzymes due to its enzymatic kinetics.

In recent decades, in silico ADME(T) modelling as a tool for rational drug design has received considerable attention from pharmaceutical scientists, and various ADMET-related prediction models have been reported. The effectiveness of these tools is highly dependent on their capacity to cope with needs at different stages of a drug development program, e.g. currently their use in candidate selection has been limited due to the fact that they lack the required predictability. Nonetheless, for some endpoints involving more complex mechanisms, the current *in silico* approaches still need further development (Kirchmair et al., 2012; Raunio et al., 2015; Wang et al., 2015).

In summary, currently modeling methods are not mature enough to replace standard *in vitro* and *in vivo* approaches, but they are already incorporated as an important component today's rational drug design programs. The high-throughput and low-cost nature of these models permits a more streamlined drug design and development process in which the identification of hits or their structural optimization can be guided based on a parallel investigation of the bioavailability and safety, along with activity. For example, in 2004, it is estimated that about 50 NCEs had reached clinical trials and/or market approval guided by structure-based drug design (Kirchmair et al., 2012; Raunio et al., 2015).

3 Aims of the Study

The general objective of this study was to gain insights into interactions of plant isoquinoline alkaloids and herbal medicine alkaloids with CYP enzymes. Another general objective was to respond to the current challenge(s) in the field of early drug interaction studies, especially concerning time-dependent CYP inhibition. The specific aims were as follows:

1. To assess the inhibitory activity of plant isoquinoline alkaloids towards all principal human drug metabolizing CYP enzymes *in vitro*
2. To develop a new fluorometric method for the assessment of CYP2C19 enzyme inactivation kinetics *in vitro*
3. To determine CYP2C19 inhibition mechanisms of three representative herbal medicine alkaloids *in vitro*
4. To examine the effect of pre-incubation on CYP2C19 inhibition by isoquinoline alkaloids *in vitro*
5. To study the interactions of isoquinoline alkaloids with CYP2C19 enzyme *in silico* and to create 3D-QSAR model
6. To evaluate the incidence of isoquinoline alkaloids in TCM

4 Materials and Methods

4.1 CHEMICALS AND ENZYMES

The following isoquinoline alkaloids were a kind gift from Dr. Prof. Peter Imming and his research group (Institute of Pharmacy, Martin-Luther-Universität Halle-Wittenberg, Germany): protopine, hunnemannine, cryptopine, allocryptopine, corycavidine, corycavamine, dihydrocryptopine, dihydroprotopine, 2,3-Dimethoxy-7-methyl-9,10-dimethoxy-5,6,7,8,13,14-hexahydrodibenzo [d,h,] azecine (AP25), apomorphine, bulbocapninemethylether, bulbocapnine, isocorydine, corydine, nantenine, parfumine, capnoidine, canadine, nandinine, corydaline, tetrahydropalmatine, corypalmine, stylopine, thalictricavine, isocorybulbine, corybulbine, scoulerine, mecambridine, 2,3,10,11-tetramethoxyberbine (norcoralydine), 8-ethyl-2,3,10,11-tetramethoxyberbine (8-ethyl-norcoralydine), 8-methyl-2,3,10,11-tetramethoxyberbine (coralydine), 8-methyl-2,3,10,11-tetraethoxyberbine (ethaverine-coralydine), 2,3-dimethoxy-10,11-methylenedioxyberbine (tetrahydropseudoepiberberine), 2,3-dimethoxy-9,10-methylenedioxyberbine (sinactine), 3,10,11-trimethoxyberbine, 3-methoxyberbine, 2,3-dimethoxyberbine, 3-hydroxyberbine, 2,3,9,10-tetrahydroxyberbine HBr (AP26), 3,10-dimethoxy-11-hydroxyberbine (AP60) (**I**, **III**, **IV**). A more detailed description of the compounds is provided in original paper **I** and in a publication by Meyer and Imming (2011).

Alkaloids bicuculline, noscapine, berberine, palmatine, chelidonine, papaverine, sinomenine, norlaudanosine, pavine (**IV**) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isoniazid, ticlopidine and *trans*-2-phenylcyclopropylamine hydrochloride (tranylcypromine) (**II–IV**) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest purity available. Fluorescein benzyl ester and fluorescein benzyl ether were synthesized in the Unit of Pharmaceutical Chemistry, University of Eastern Finland (**II**).

cDNA-expressed human wild-type CYP enzymes (Supersomes™) (**I–IV**) were obtained from BD Biosciences Discovery Labware (Bedford, MA, USA).

4.2 TCM DATABASE SEARCH (I, IV)

A search of a commercial TCM Database: Traditional Chinese Medicines: Molecular Structures, Natural Sources and Applications (NiceData, 2005) was carried out based on names, structures and CAS numbers of the alkaloids. This database includes 10458 individual compounds found in TCM plants, 4636 TCM medicinal plants and their therapeutic utilities.

4.3. IN VITRO CYP INHIBITION SCREENING (I)

Inhibition of the main human drug metabolizing CYP enzymes 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 was studied using traditional endpoint assays originally published by Crespi et al. (1997) and described in detailed in original paper **I**. Incubations were performed in 96-well microtiter plates, using cDNA-expressed recombinant CYP enzymes and four or five concentrations of test compounds at a 1:10 ratio ranging from 0.01 to 1000 μM. The incubation mixtures contained 100 mM Tris/HCl buffer (pH 7.4), 0.75–2.0 pmol of CYP enzyme, the probe substrate at the concentration matching its measured apparent K_m, and the NADPH-regenerating system, except that 50 mM Tris/HCl buffer (pH 7.4), 0.3 mM NADPH, and 5 mM $MgCl_2$ in a 100 μl incubation volume was used in the case of CYP2A6. If the test compounds were not soluble in water, they were dissolved in dimethyl sulfoxide (DMSO) or acetonitrile (ACN) and then further diluted with water. The final solvent concentrations were not above 2%. Controls were treated correspondingly but without the test compound. The reactions were initiated by adding 50 μl of the NADPH-regenerating system (NADPH in the case of CYP2A6), after a 10-min preincubation at 37°C. After incubation (10–60 min), the reactions were stopped by addition of 80% ACN/20% 0.5M Tris in the assays of 1A2, 2B6, 2C9, 2D6, and 3A4; 2 M NaOH in the assays of 2C8 and 2C19; and 10% TCA in the 2A6 assay. In the CYP2A6 assay, 140 μl of 1.6M glycine–NaOH buffer (pH 10.4) was added into the wells immediately before the measurement.

Fluorescence was determined with a Victor2 plate counter (Perkin-Elmer Life Sciences Wallac, Turku, Finland) with excitation and emission wavelengths adjusted to the probe substrate/metabolite. The IC_{50} values (inhibitor concentration reducing the rate of metabolism by 50%) were calculated using non-linear regression analysis with Prism 4.0 software (San Diego, CA, USA).

4.4 CYP2C19 PROGRESS CURVE ANALYSIS METHOD (II–IV)

Liquid chromatography (LC)-electrospray ionization-mass spectrometry (MS) methods were used to evaluate the properties of dibenzylfluorescein (DBF) as a substrate for fluorescence-based CYP2C19 activity and inhibition assays. Fluorescein benzyl ester and fluorescein benzyl ether synthesized in-house along with commercial fluorescein were used as standards. Further, requirements for a real-time kinetic assay were evaluated by determining whether enzyme activity could be detected without using 2 M NaOH as a stop solution. In addition, CYP2C19 reaction linearity with respect to enzyme concentration and incubation time was investigated by using different enzyme amounts (0.125–2 pmol). The detailed experimental conditions and procedures are described in original paper **II**.

Real-time kinetic assay and progress curve analysis assay comprise an “all-in” approach in which the enzyme (1.5 pmol of cDNA-expressed recombinant CYP2C19) is exposed concomitantly with the probe substrate DBF (1 μM), inhibitor or the vehicle, and (50 μl of) the NADPH-regenerating system in a 150 μl total volume in microplate wells. Incubations were conducted at pH 7.4 (100 mM Tris/HCl buffer) and at 37°C in a Victor2 plate scanner. Controls were handled similarly but without adding the inhibitor and the blanks did not contain the enzyme. Real-time kinetic assays, in general, could be initiated by addition of

the substrate, enzyme or the NADPH-regenerating system. Progress curve analysis experiments were conducted by measuring first the substrate alone in a Victor2 plate scanner for 15 min at 37°C. After that, the prewarmed enzyme, inhibitor/vehicle and the NADPH-regenerating system were added and fluorescence data acquisition was initiated immediately. The enzyme activity was monitored by following continuous fluorescence intensity at 1-min intervals for 45 min (excitation 485 nm and emission 535 nm). Visual inspection of progress curves reveal TDI. TDI converts the linear progress curve seen in the absence of the inactivator into a curvilinear function. The data was analyzed to determine the key kinetic parameters and mechanistic information of the inactivation process.

For determination of IC_{50} values, the linear regions of progress curves were used to calculate reaction velocity, and the remaining activities were plotted as a function of the logarithm of the molar concentration of inhibitor. The curves were fitted to a sigmoid dose-response equation with Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For TDI, each progress curve was fitted by the following equation (Eq. 10) (Copeland, 2005), which contains terms for the initial and steady-state velocities (v_i and v_s) and for the rate constant for onset of inhibition (k_{obs}), i.e., conversion from the initial velocity phase to the steady-state velocity phase (Eq. 10). K_{obs} values were then plotted against inactivator concentrations and fitted to equation 11 (Fairman et al., 2007).

Eq. 10

$$[\text{Product}] = v_s t + \frac{v_i - v_s}{k_{obs}}\left[1 - \exp(-k_{obs} t)\right]$$

Eq. 11

$$k_{obs} = \frac{k_{inact}[\text{I}]}{K_I(1 + \text{S}/K_m) + [\text{I}]}$$

Progress curve experiment was first applied to evaluate two known mechanism-based (time-dependent) inactivators, isoniazid and ticlopidine, and one known reversible inhibitor, tranylcypromine. Seven different concentrations of each test compound at a 1:3 ratio were used to ensure a wide range of inactivation during the incubation measurement. Isoniazid and ticlopidine were dissolved in water, and tranylcypromine was dissolved in acetonitrile and then further diluted with water. The final solvent concentrations were not above 2.2%. Controls were treated in the same way but without the presence of inhibitors.

4.5 IN VITRO TDI SCREENING FOR CYP2C19 (III, IV)

IC_{50} shift assay. Real-time kinetic assay procedure was used with 30-min preincubation. A mixture containing the inhibitor (at seven different concentrations, mainly 1:3 ratio), enzyme (1.5 pmol of cDNA-expressed recombinant CYP2C19), and the NADPH-regenerating system was first preincubated for 30 minutes at 37°C. Subsequently, the reactions were initiated by adding the substrate (1 μM DBF). Controls were treated in a similar way but without addition of inhibitor and blanks without the enzyme. For determination of IC_{50} values, the same procedure was used as described in section 4.4. IC_{50}

values were compared with those obtained in similar experimental conditions without preincubation as measured according to section 4.3.

Progress curve analysis assay. Experiments were conducted as described in section 4.4 using seven different concentrations of each alkaloid and substrate added before the measurement. The inhibitor concentrations were selected to ensure a wide range of inactivation during the 45-min incubation measurement. Controls were treated in a similar way but without addition of inhibitor and blanks without the enzyme. The curves were first visually inspected to determine whether a compound exhibited time- and concentration-dependent inhibition, after which the data obtained were analyzed to determine the key kinetic parameters (k_{obs}, k_{inact}, and K_I) and saturation kinetics on the inactivation process.

4.6 IN VITRO MBI CRITERIA EXPERIMENTS (III)

Dialysis experiment. A dialysis experiment was conducted to evaluate whether removal of unbound inhibitor from the enzyme solution would reverse the CYP2C19 inactivation. A single concentration (that caused >90% inactivation) was used for each test compound and was incubated with CYP2C19 at 37°C for 30 min before dialysis in the presence or absence of the NADPH-regenerating system. Controls were treated in a similar way but with no inhibitors included. The incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), the inhibitor (at concentration causing >90% inactivation), 0.1 μM CYP2C19, and the NADPH-regenerating system or the vehicle. The samples were dialyzed after the 30-min incubation in Slide-A-Lyzer mini-dialysis units (molecular weight cutoff 3500; Thermo Fisher Scientific, Waltham, MA) against 2.0 liters of 0.1 M potassium phosphate buffer, pH 7.4 (four 500-ml treatments for 2 h each) for 10 to 12 h at 4°C. Equivalents before the dialysis and from the dialyzed samples were pipetted into 96-well plates for kinetic CYP2C19 activity measurements. The reactions were initiated by adding the NADPH-regenerating system and the substrate (1 μM DBF), after which the reactions were measured at 1-min intervals for 60 min. Isoniazid, a well-known mechanism-based inactivator, was used as a positive control, and tranylcypromine, a recognized reversible inhibitor, was used as a negative control.

Spectroscopic determination of MIC formation. Recombinant CYP2C19 was used to characterize the MIC formation associated with the metabolism of test compounds. Tranylcypromine, a known reversible inhibitor, was used as a negative control. The incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), the inhibitor (at inhibition concentration), 0.13 μM CYP2C19 enzyme and the NADPH-regenerating system, while the reference sample contained the vehicle that was used to dissolve the inhibitor. All MIC formation experiments were initiated by adding the NADPH-regenerating system and incubated at 37°C for 15 min. MIC formation was observed with an EnVision 2104 multilabel plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA) by scanning from 400 to 500 nm to monitor changes in the absorbance spectra. Identical assay procedure but in the absence of the NADPH-regenerating system was also conducted. Data were analyzed using Prism 5.0 software (GraphPad Software Inc., San Diego, CA).

4.7 MOLECULAR DOCKING AND 3D-QSAR STUDIES (IV)

Structure preparation. In the molecular docking studies, the crystal structure of human CYP2C19 with the inhibitor 2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl)methanon (PDB: 4GQS) (Reynald et al., 2012) was used. The protein structure was processed with the Protein Preparation Wizard of Schrödinger Maestro v. 9.6 (Schrödinger, 2013) using standard settings (add hydrogens, assign bond orders, create zero order bonds to metals and disulfide bonds and delete waters beyond 5 Å from heteroatoms). Hydrogen bonds were assigned and the prepared structure was minimized using OPLS_2005 force field and restrained minimization (heavy atom converging RMSD 0.30 Å). The lacking side chains were added to the crystal structure using Prime version 3.0 (Schrödinger, 2012a).

Small molecules were preprocessed with LigPrep v. 2.5 (Schrödinger, 2011); the molecules were desalted, tautomers were determined at pH 7 ± 1 using Epik and all possible enantiomers (or a maximum of 32) were created if chirality was not present in the original structures.

Molecular docking. Alkaloids (N=49) were docked in the active site of CYP2C19 using Glide version 5.8 (Friesner et al., 2004; Halgren et al., 2004; Friesner et al., 2006; Schrödinger, 2012b). The Glide grid-file was constructed for ligands within 20 Å in length and the center of the grid was defined with the co-crystallized ligand (2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl)methanon) of the crystal structure of CYP2C19 (PDB: 4GQS). The metal coordination constraint of heme iron as well as the positional constraints that define the spherical region occupied by inhibitors were applied. The positional constraint was defined into the putative binding region of canadine (**20**). The flexible docking studies were carried out for all alkaloids. The docking results were visually inspected, and the highest scoring docking poses displaying orientation toward the heme moiety were chosen for 3D-QSAR evaluation using the Comparative Molecular Field Analysis (CoMFA) method with Sybyl-X 2.0 (Cramer et al., 1988; Tripos, 2011).

CoMFA models. Molecular docking was used to align 49 alkaloids in their biologically active conformation. The alkaloids were divided into a training set (N=37) and a test set (N=12). The test set contained alkaloids with diverse activities (IC_{50} 1.4–150 μM) and structures. For the alkaloids the steric and electrostatic fields were calculated. Multiple partial least square (PLS) models were built and validated using the cross-validation and progressive scrambling methods to obtain the optimum number of PLS components for the final analysis. The leave-one-out (LOO) and cross-validation models with different numbers of components (1–5 components) and with different group sizes (two and five groups) were carried out with column filtering set to 2.0. The final analysis was done with three components.

5 Results and Discussion

5.1 TCM DATABASE SEARCH (I, IV)

To examine whether the current alkaloids (N=49) are present in TCM and to evaluate their potential to be ingested by humans, a search of a commercial TCM Database was carried out. Of the 49 alkaloids, 26 (53%) were found in the database. These 26 alkaloids were present in several TCM plants and medicinal plants. For 15 alkaloids (**1**, **3**, **4**, **12**, **13**, **14**, **17**, **20**, **30**, **31**, **43**, **44**, **45**, **46**, **47**) one or more TCM pharmacological or therapeutic effects were listed. All alkaloid numbers are according to original paper **IV**. In addition, based on the search, three of the mentioned plants (in original paper **I**) were also detected (*Papaver somniferum L.*, *Chelidonium majus L.*, and *Corydalis cava L.*). Search results with information from the commercial TCM Database are collated in Tables 6 and 7 (at the end of this thesis). Examples of molecular structures of the studied alkaloids are presented also in Figure 11.

At least 28 of the studied alkaloids (26 plus apomorphine and noscapine) are likely to be ingested by humans. The exposure is possible via conventional medicines (in the cases of apomorphine **10**, noscapine **19**, papaverine **46**) or via herbal preparations. In addition to these 28 alkaloids, five plant-derived alkaloids examined here (hunnemannine **2**, corycavidine **5**, parfumine **16**, thalictricavine **26** and isocorybulbine **27**) were not found in the database, but for those, however, human exposure is possible via products made from the following plants: *Corydalis cava* L., *Hunnemannia fumariifolia* Sweet of *Fumaria vaillantii Loisel*.

All alkaloids in this research project have aroused considerable medicinal and pharmaceutical interest, as benzylisoquinoline alkaloids in general are under intensive research for medicinal purposes. *Papaver somniferum* (a source of at least alkaloids **1**, **3** and **46**) is one of the world's oldest medicinal plants and it still remains the only commercial source for many important alkaloids. *Chelidonium majus* L. (a source of at least alkaloids **1**, **3**, **4**, **25**, **43** and **45**) and *Corydalis cava* L. (a source of at least alkaloids **5**, **6**, **12**, **13**, **14**, **15**, **17**, **22**, **23**, **24**, **25**, **26**, **27**, **28** and **29**) have also a long history as being useful for the treatment of many diseases in European countries and in Chinese herbal medicine (Colombo and Bosisio, 1996; Gilca et al., 2010; Nawrot et al., 2014). In addition, the European Pharmacopoeia (Ph. Eur.) includes *Fumaria officinalis* L. This herb and its phytotherapeutic tablets contain protopine (**1**) and cryptopine (**3**) as major isoquinoline alkaloids (Gotti, 2011). In addition, exposure to berberine (**43**) and canadine (**20**) is possible via the herbal medicinal product goldenseal (Hermann and von Richter, 2012).

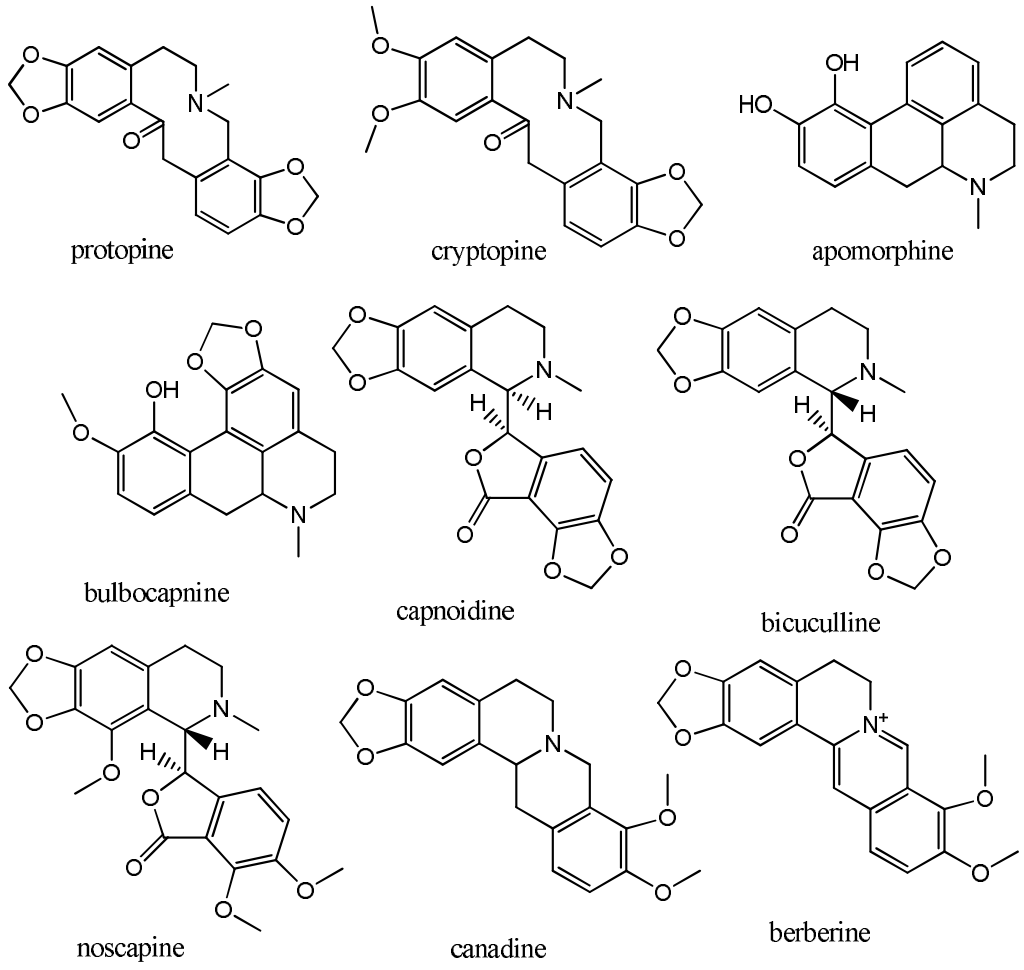


Figure 11. Examples of molecular structures of the studied isoquinoline alkaloids (Figures 1 and 3).

5.2 IN VITRO CYP INHIBITION SCREENING (I)

Inhibition of main human drug metabolizing CYP enzymes 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 by 36 plant isoquinoline alkaloids were studied using fluorescent rhCYP endpoint assays. Based on the extent of inhibition, alkaloids were classified as potent (IC_{50} < 1 μM), marginal/moderate (1μM < IC_{50} < 10μM), weak (IC_{50} > 10μM), or non-inhibitors (IC_{50} > 100 μM) (White, 2000). The results of this study provided a good perspective of the interactions between novel plant isoquinoline alkaloids and the most important human CYP enzymes. The current study revealed the presence of high-affinity interactions of several isoquinoline alkaloids with several CYP enzymes (**I**: Table 1). This is not surprising taking into account the fact that enzymes involved in BIA metabolism in plants belong to a relatively limited number of protein families, including CYP enzymes (Beaudoin and Facchini, 2014). However, clear differences in the ability of the plant isoquinoline alkaloids (N=36) to inhibit individual CYP forms were observed. The screening results are summarized in Table 8 and Figure 12 below. The tested isoquinoline alkaloids inhibited most potently the CYP3A4 form (30/36 with at least moderate potency and 15/36 potently),

and CYP2D6 (26/34 with at least moderate potency and 21/34 potently), and to some extent also CYP2C19 (15/36 with at least moderate potency and 3/36 potently). Protopine and protoberberine alkaloids were more potent inhibitors of CYP2D6 than CYP3A4. In contrast to CYP2D6, CYP3A4 was inhibited by the aporphine alkaloids. **Alkaloids inhibiting CYP2C19 mostly belonged to the protopine and protoberberine alkaloids**; none of the aporphine alkaloids displayed potent inhibition.

The alkaloids had a clearly less potent inhibitory effect on CYP2C8 (3/36 with moderate potency), CYP2B6 (4/36 with moderate potency, all of them protopine alkaloids), and CYP2C9 (6/36 with moderate potency). In addition, 10/36 alkaloids inhibited CYP1A2 with moderate potency and 1/36 (nantenine) potently. None of the studied alkaloids showed any ability to inhibit CYP2A6.

Table 8. Results of CYP inhibition screening and TDI screening for CYP2C19 assays *in vitro* according to the classification of the inhibition potencies of the alkaloids.

Inhibition category	3A4	2D6	Pre-incubated 2C19	2C19	1A2	2C9	2B6	2C8	2A6
Potent (IC_{50} < 1μM)	15/36	21/34	9/49	3/49	1/36	0	0	0	0
Moderate (IC_{50} 1–10μM)	15/36	5/34	15/49	13/49	10/36	6/36	4/36	3/36	0
Weak (IC_{50} 10–100μM)	4/36	7/34	19/49	20/49	23/36	14/36	19/36	17/36	1/36
Non-inhibitor (IC_{50} >100 μM)	2/36	1/34	6/49	13/49	2/36	16/36	13/36	16/36	35/36
% (IC_{50} ≤10 μM)	84	77	49	32	31	16	11	8	0

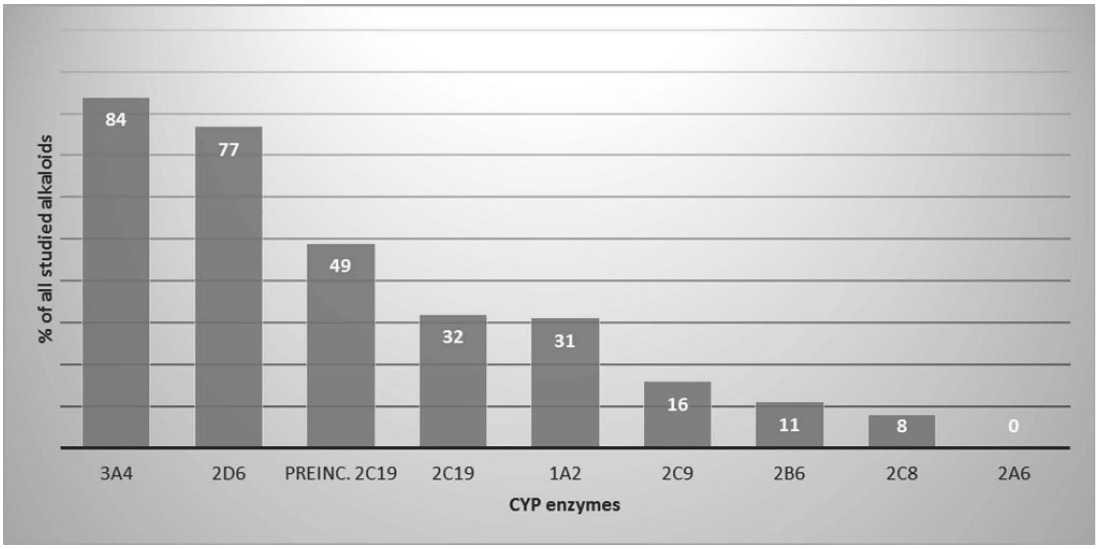


Figure 12. CYP inhibition by isoquinoline alkaloids, proportions of potent plus moderate potent inhibitors (IC_{50} < 10 μM) of all.

Several new potent inhibitors against CYP3A4, CYP2D6, CYP2C19 and CYP1A2 were found. Of particular interest was the selective inhibition of CYP3A4 by corydine, parfumine and 8-methyl-2,3,10,11-tetraethoxyberbine (Figure 13).

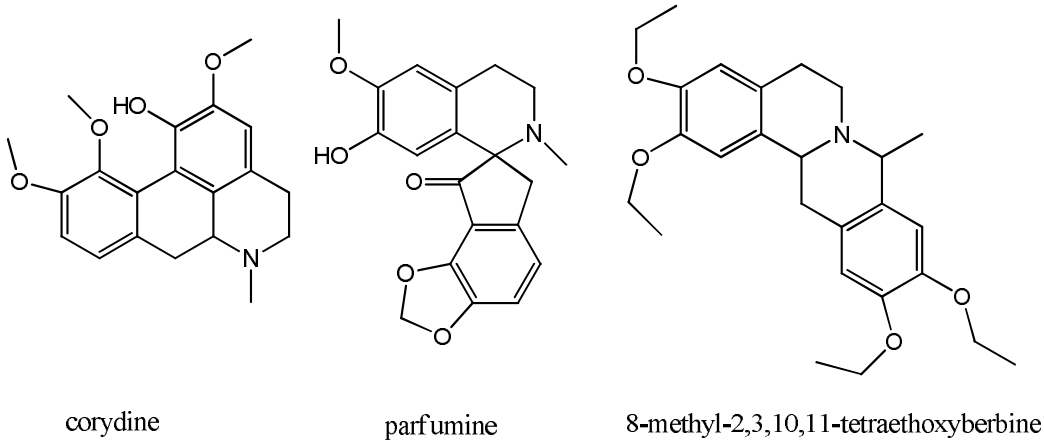


Figure 13. CYP3A4 selective inhibitors in the CYP inhibition screening study.

The tested isoquinoline alkaloids possessed typical CYP2D6 substrate properties: lipophilic bases with a planar hydrophobic aromatic ring and a nitrogen atom which can be protonated at physiological pH (Lewis, 2004).

When considering these kinds of isoquinoline alkaloids in drug discovery and development, some conclusions can be drawn: special attention should be paid to their effects towards the most important CYPs, especially CYP3A4 and the polymorphic CYP2D6 and CYP2C19. These alkaloids are potential substrates for the highly polymorphic CYP2D6 as well as for CYP3A4. Based on this inhibition study, it is not possible to conclude whether these alkaloids are true inhibitors or simply substrates of CYP2D6 and CYP3A4. However, in recent years, pharmaceutical companies have tried to avoid developing drug candidates that are substrates or inhibitors for the highly polymorphic CYPs such as CYP2D6 and CYP2C19, because it has been reported that almost half of the drugs involved in the appearance of adverse drug reactions are metabolized by polymorphic CYP enzymes (Singh et al., 2011). On the other hand, based on this study (i.e., affinities for several CYPs), these alkaloids might have potential to be metabolized via several CYP enzymes.

As far as is known, the literature does not contain other wide-ranging research on CYP inhibition by pure isoquinoline alkaloids. However, some CYP inhibition studies have been performed with individual isoquinoline alkaloids e.g. corynoline (Fang et al., 2011; Mao et al., 2015); sanguinarine (Qi et al., 2013); noscapine (Zhang et al., 2013); tetrahydropalmatine, neferine and berberine (Zhao et al., 2015). CYPs 2C9, 3A4 and 2C19 were found to be mainly involved in metabolic activation of corynoline to reactive metabolites (Mao et al., 2015); sanguinarine has been identified as inhibitor for CYPs 1A2, 2C8, 2C9 and 3A4 (Qi et al., 2013); MBI of CYP2C9 by noscapine was observed (Zhang et al., 2013); and finally, tetrahydropalmatine, neferine and berberine inhibited 1A2, 2D6 and 3A4 mainly in a MBI manner (Zhao et al., 2015).

Wu et al. (2012) have reviewed interactions between phytochemicals from TCM and CYP enzymes. Several alkaloids and other natural products have been identified as substrates, inhibitors or inducers of CYP enzymes. For example, the isoquinoline alkaloids, berberine and corynoline, were identified as substrates for CYP1A2, CYP2D6 and CYP3A4 enzymes and CYP2C9 and CYP3A4 enzymes, respectively, as well as inhibitors for CYP2E1 (berberine and hydrastine) and for CYP2C9 and CYP3A4 (corynoline).

The herbal medicinal product, goldenseal, contains rather many isoquinoline alkaloids (such as hydrastine, berberine, berberastine, hydrastinine, tetrahydroberberastine, canadine and canalidine, of which hydrastine and berberine are the main active constituents) and are reported to inhibit CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 enzymes (Shi and Klotz, 2012).

The current evidence suggests that, in general, *in vitro-in vivo* metabolic correlations are plausible in qualitative terms, however, *in vitro* extrapolations can be confounded by various *in vivo* biochemical and biophysical factors making it difficult to express the risk in quantitative terms (Wienkers and Heath, 2005; Pelkonen et al., 2011). Although HLM assays have been considered as the gold standard, *in vitro* models using recombinant CYPs are extremely useful when examining structure-activity relationships. Fluorometric inhibition screening assays have been found to be comparable with other potential screening procedures used, such as HPLC and mass spectrometry detection (Turpeinen et al., 2006; Pelkonen et al., 2008; Kapitulnik et al., 2009). Nonetheless, it has also been reported that the recombinant CYP-fluorescent assay generated more false-positive results than HLM-based assays when compared to CYP inhibition data obtained from clinical studies (Bell et al., 2008).

5.3 DEVELOPMENT OF THE CYP2C19 PROGRESS CURVE ANALYSIS METHOD (II)

DBF is widely used as a profluorescent probe substrate for CYP activity and inhibition assays (for example for 2C8, 2C9, 2C19, 3A4, and aromatase (CYP19)), but its use has been thought to be limited to traditional endpoint assays. The utility of DBF in kinetic assay procedures has been questioned because the initial metabolite of DBF (fluorescein benzyl ester) requires very alkaline conditions for further hydrolysis to fluorescein in order to maximize the fluorescence intensity (Crespi and Stresser, 2000; Miller et al., 2001).

Initially, the prerequisites for kinetic assay procedure were examined. Those were; CYP2C19-catalyzed metabolism of DBF, the effect of the base on fluorescence of DBF and its metabolites and the stability of their fluorescence intensities, and signal-to-noise ratios in determinations of enzyme-catalyzed reactions.

In the CYP2C19-catalyzed reaction, fluorescein benzyl ester was formed as a major metabolite. In addition, minor amounts of fluorescein benzyl ether were also detected, but fluorescein was not formed at pH 7.4. In the blank samples, without the enzyme, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein were not formed spontaneously from DBF. Addition of 2 M NaOH caused the decomposition of fluorescein benzyl ester to fluorescein and the decomposition of DBF to fluorescein benzyl ether whereas fluorescein benzyl ether and fluorescein remained unchanged in the presence of 2 M NaOH (Figure 14). In the absence of 2 M NaOH, the standard compounds remained

unchanged. The results of signal-to-noise ratio determinations showed that it is feasible to detect enzyme activity without the use of 2 M NaOH as the signal to noise ratio was ~12-fold without the use of 2 M NaOH (**II**: Table 3). In addition, 15-min premeasurement of DBF substrate enabled detection of linear reaction kinetics from the very beginning of the enzyme reaction (**II**: Figure 3). As a conclusion, DBF is suitable for use in kinetic assays and progress curve analysis approaches.

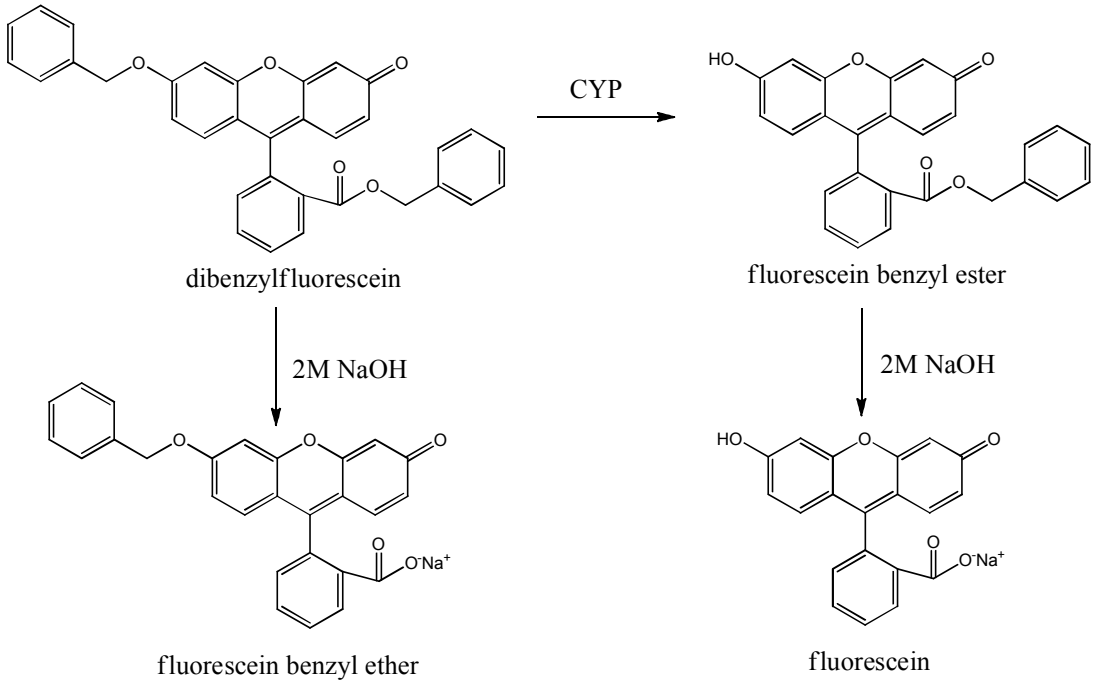


Figure 14. Structures of a probe substrate DBF and fluorescent products. DBF is dealkylated by CYP2C19 to form a fluorescein benzyl ester. It is further hydrolyzed to fluorescein by addition of 2 M NaOH. Addition of 2 M NaOH causes decomposition of DBF to fluorescein benzyl ether.

Subsequently, the use of DBF in a kinetic assay as a progress curve analysis was demonstrated to achieve a straightforward determination of whether a compound is a time-dependent inactivator of CYP2C19. The progress curve experiment was first applied to evaluate two known mechanism-based and time-dependent inactivators, isoniazid and ticlopidine, and one known reversible inhibitor, tranylcypromine.

Isoniazid is used for the treatment and prevention of tuberculosis and ticlopidine is clinically utilized as an anti-platelet aggregation agent. Clinically relevant interactions between these drugs and substrates of CYP2C19 have been reported and they are confirmed to occur via mechanism-based inactivation (Donahue et al., 1997; Tateishi et al., 1999; Nishimura et al., 2003; Richter et al., 2004; Kalgutkar et al., 2007). Isoniazid and ticlopidine exhibited clear time-dependent inactivation (Figure 15) and saturation in their inactivation kinetics (Figure 16) with K_I and k_{inact} values of 250.5 ± 34 μM and 0.137 ± 0.006 min^{-1} and 1.96 ± 0.5 μM and 0.135 ± 0.009 min^{-1}, respectively. The determined inactivation kinetic constants (K_I and k_{inact}) correlated well with published data, confirming the reliability of the method (Table 9).

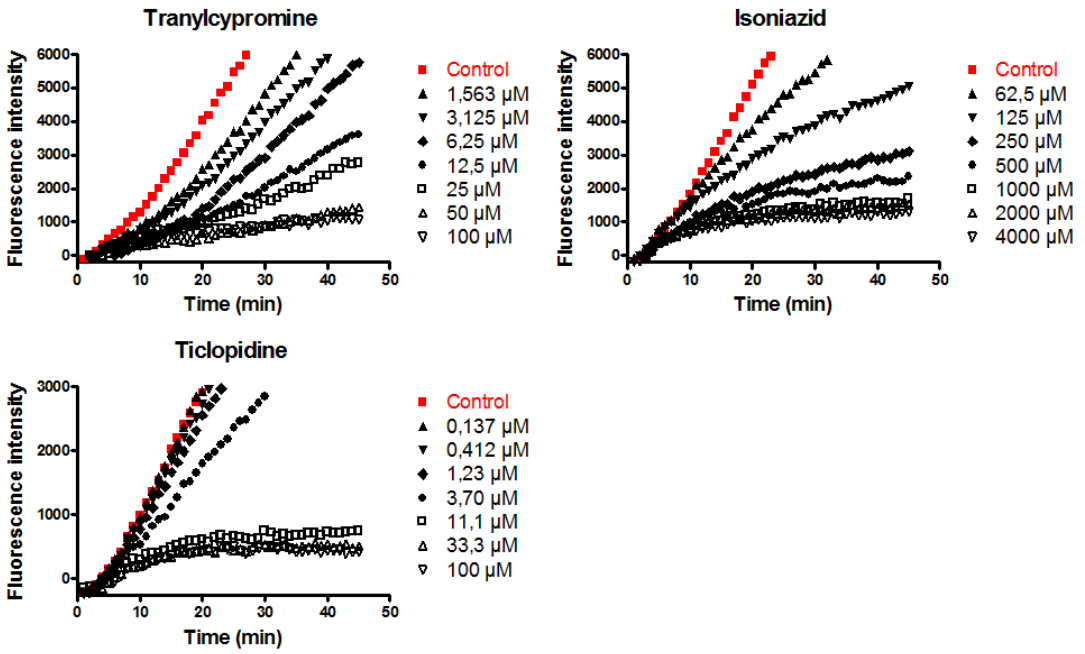


Figure 15. Progress curves of CYP2C19 inhibition by tranylcypromine, isoniazid and ticlopidine.

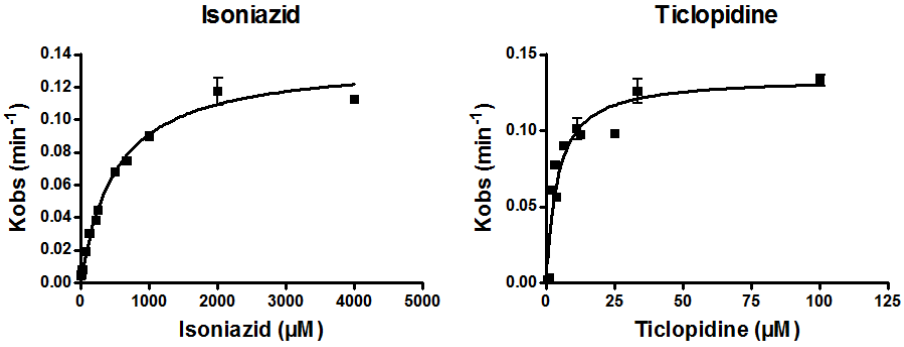


Figure 16. Plots of k_{obs} against inactivator concentration for isoniazid and ticlopidine indicated saturation at higher concentrations, pointing to a two-step inactivation mechanism.

Table 9. Inactivation kinetic constants for isoniazid and ticlopidine.

	Determined		**Published data**			
Inactivator	**K_I (µM)**	**k_{inact} (min^{-1})**	**K_I (µM)**	**k_{inact} (min^{-1})**	**Enzyme source and substrate**	**Reference**
Isoniazid	250.5 ± 34	0.137 ± 0.006	112	0.090	HLM and S-mephenytoin	(Wen et al., 2002)
			255	0.020	HLM and S-mephenytoin	(Nishimura et al., 2003)
			79.3	0.039	HLM and S-mephenytoin	(Polasek et al., 2006)
Ticlopidine	1.96 ± 0.5	0.135 ± 0.009	1.65	0.192	HLM	(Venkatakrishnan and Obach, 2007)
			4.30	0.097	-	(Kalgutkar et al., 2007)
			3.32	0.074	HLM and S-mephenytoin	(Nishiya et al., 2009)

Tranylcypromine did not display TDI (Figure 15), which is consistent with its reported mechanism of reversible competitive inhibition of CYP2C19. The competitive inhibitor, tranylcypromine, is frequently used as positive control compound in CYP2C19 inhibition studies (BD Gentest, 2000; Lin et al., 2007). Tranylcypromine is a nonselective irreversible inhibitor of monoamine oxidase (MAO) and has been used for the treatment of depression. Its inhibitory effects are not considered likely to cause clinically significant interactions at the usual therapeutic doses (Salsali et al., 2004; Polasek et al., 2006).

In summary, DBF can be used as a substrate both to screen and to test compounds that require more detailed investigation. The full progress curve of an enzymatic reaction contains an abundance of valuable kinetic information and allows investigation of both reversible and irreversible inhibition mechanisms and components, providing more information in one experiment. Due to its simplicity and low cost, this progress curve analysis assay can be easily implemented as a high/semi-high throughput method screening for a large number of compounds in drug discovery. Despite those advantages, the fluorescence-based progress curve analysis assay is subject to some major limitations. It is worth noting that the recombinant CYP model system does not reflect the complexity of the *in vivo* scenario in which multiple enzymes co-exist and function sequentially or in parallel. In addition, the fluorescent substrates, including DBF, are not real drugs and lack CYP-selectivity. Thus they cannot be utilized in studies using HLM or in *in vivo* approaches.

Although progress curve methods for the assessment of MBI were first described over 30 years ago (Waley, 1980; Garcia-Canovas et al., 1989; Wimalasena and Haines, 1996), this has remained a relatively unexploited method for the characterization of TDI and MBI with respect to drug-metabolizing enzymes. Fairman et al. (2007) described a progress curve analysis method for HLM using LC-MS as an analytical method. The fluorometric CYP2C19 progress curve analysis described here is another application of the progress curve method in the field of CYP enzymes. The use of fluorescence assay is advantageous because one can monitor "progress" of the curve in real-time and measure the reaction directly from the reaction wells without separate analytical methods thus offering a more efficient screening and lower costs. However, there is less flexibility in the choice of substrates and enzyme source.

The distinction between reversible inhibition and MBI can be difficult to determine if the inhibitor binds tightly to the enzyme and is released slowly. In addition, one limitation of progress curve analysis approaches, and similar high-throughput methods (Yan et al., 2002; Naritomi et al., 2004), is that TDI may also arise from the generation of potent reversible inhibitory metabolites. However, TDI data should never be over-interpreted as MBI, and experiments to establish irreversibility are necessary in each case. With these caveats, TDI represents a useful screening approach for identifying potential mechanism-based inactivators. Because of the relatively recent introduction of the progress curve method, the ability of this assay design to predict DDI is not yet established (Stresser et al., 2014).

Conventional *in vitro* inactivation studies for MBI use a preincubation step followed by dilution with a probe substrate to evaluate remaining CYP activity. The dilution step will minimize the contribution of reversible inhibitors. New methods are needed, because these complex *in vitro* inactivation research methodologies have been criticized for being influenced by a wide range of experimental conditions (i.e., preincubation time, dilution factor and activity measurement time), complicating comparison across studies and making

it difficult to make clinical predictions. In addition, these conventional experiments offer limited mechanistic insights (Ghanbari et al., 2006; Riley et al., 2007; Fowler and Zhang, 2008; Obach, 2009; Zhou and Zhou, 2009).

In general terms, the ability to use *in vitro* human CYP TDI data for *in vivo* drug interaction predictions should be viewed as a prerequisite to generate the data (Grime et al., 2009). Important parameters in making such predictions are K_I and k_{inact} but first-line screening assays typically involve determination of an IC_{50} value or a time dependent shift in IC_{50}. Progress curve analysis assays with known MBI compounds (isoniazid and ticlopidine) were used to determine the desired K_I and k_{inact} values reliably (Table 9). From that point of view, this study can be viewed as a preliminary step in this research area. The present method development is above all a further development of an existing method, responding to the current needs and challenges in this research field. However, more reversible and irreversible inhibitors are needed to validate this assay for both TDI screening and DDI predictions.

5.4 IN VITRO TDI SCREENING FOR CYP2C19 (IV)

Ultimately, 42/49 (86%) of the studied alkaloids were confirmed to demonstrate TDI of CYP2C19 activity using either the IC_{50} shift assay or the progress curve analysis assay and were classified as time-dependent inhibitors (**IV**: Table 1). For alkaloids **2**, **27**, **31**, **33**, and **34** their insufficient solubility made it impossible to obtain reliable results for these compounds and **15** and **24** were not available for TDI analysis. For most alkaloids (i.e., 39 of 49, 79.6%), the IC_{50} value decreased ≥1.5-fold if there was a preincubation. As a result, in the cases of 16 alkaloids, the classification changed to being more potent. This meant also that some alkaloids classified initially as non-inhibitors exhibited inhibition after preincubation. The known time-dependent inhibitor isoniazid and the known reversible inhibitor tranylcypromine were used as control compounds. As expected, the inhibition potency of isoniazid was enhanced (the IC_{50} value of isoniazid reduced up to 10-fold) whereas that of tranylcypromine was not enhanced by preincubation. In fact, the inhibition potency was slightly reduced in the case of tranylcypromine (the IC_{50} value of tranylcypromine doubled).

For alkaloids **14**, **17**, **22**, **37**, **38**, and **39**, time- and concentration-dependent inhibition was confirmed by the progress curve analyses. In addition, K_I and k_{inact} values were calculated for **17** (2.8 ± 0.69 μM and 0.144 ± 0.009 min^{-1}), **22** (68 ± 23 μM and 0.286 ± 0.057 min^{-1}), **38** (77 ± 18 μM and 0.129 ± 0.017 min^{-1}), and **39** (358 ± 89 μM and 0.094 ± 0.010 min^{-1}) and saturation in their inactivation kinetics observed.

Nine of the studied alkaloids were found to be potent, 15 moderately potent and 19 weak inhibitors. Only six were classified as non-inhibitors. Alkaloids inhibiting CYP2C19 belonged mostly to the protopine and protoberberine alkaloids; none of the aporphine alkaloids exhibited potent inhibition. The presence of the MDP moiety increased the inhibitory potency. All the potent inhibitors (IC_{50} < 1 μM) possessed the MDP moiety, while 50% of the moderately potent inhibitors (IC_{50} 1–10 μM) and 28% of weakly inhibitory alkaloids (IC_{50} > 10 μM) contained the MDP. Those alkaloids with two MDP moieties (n = 7) were among the most potent (IC_{50} < 2 μM) inhibitors, with the exception of bicuculline **18** (IC_{50} 150 μM). It is known that compounds containing the MDP moiety can inactivate CYP

enzymes as a result of the formation of a MIC with the enzyme, which leads to quasi-irreversible and thus time-dependent enzyme inactivation (Casida, 1970; Franklin, 1971; Nakajima et al., 1999; Correia and Ortiz de Montellano, 2005).

The observed TDI of the polymorphic CYP2C19 by isoquinoline alkaloids warrants special attention during drug discovery and development and when used clinically. Noscapine (**19**) is an illustrative example with its reported *in vivo* effects and *in vitro* findings, consistent with these research results (the potent inhibition with IC_{50} of 0.2 μM, the presence of TDI, and noscapine's orientation in the active site of CYP2C19). Noscapine is widely used as an antitussive drug and has been associated with serious drug interactions when coadministered with warfarin (Ohlsson et al., 2008; Scordo et al., 2008; Fang et al., 2010). Previously noscapine has been demonstrated to cause marked inhibition of CYP2C9 and CYP2C19 activity *in vivo* (Rosenborg et al., 2010), to inhibit at least CYP2C9 and CYP3A4 in a time-dependent fashion *in vitro* (Fang et al., 2010), and to be a mechanism-based inhibitor of CYP2C9 *in vitro* (Zhang et al., 2013).

The IC_{50} shift assay is a straightforward and commonly applied test to discover TDI, whereas the progress curve analysis method has been used more recently for CYP enzymes. Both of these assays require further studies to confirm the TDI nature or mode of action behind the TDI (see the next section 5.5). For example, the consistent clinical observations of noscapine, isoniazid and ticlopidine support the reliability of these studies.

5.5 IN VITRO MBI CRITERIA EXPERIMENTS (III)

Specific *in vitro* criteria were used to evaluate whether three MDP compounds, bulbocapnine, canadine and protopine, would be mechanism-based inactivators of CYP2C19. They indicated time-dependent inhibition, saturation kinetics, irreversibility, NADPH dependence, and involvement of a catalytic step in the enzyme inactivation. These alkaloids were chosen to represent the largest alkaloid subgroups and different inhibition potency classes. These alkaloids are included in TCM and they occur very widely in medicinal plants. For example, protopine is a major isoquinoline alkaloid in *Fumaria officinalis* herba and its phytotherapeutic tablets (Gotti, 2011). *Fumaria officinalis* is included also in the European Pharmacopoeia (Ph. Eur.).

TDI and saturation kinetics; application of the progress curve analysis. In this experiment bulbocapnine, canadine and protopine exhibited time- and concentration dependent inhibition and saturation in their inactivation kinetics (similar to those of isoniazid and ticlopidine) pointing to a two-step inactivation mechanism. K_I and k_{inact} values for bulbocapnine were 72.4 ± 14.7 μM and 0.38 ± 0.036 min^{-1}, for canadine 2.1 ± 0.63 μM and 0.18 ± 0.015 min^{-1}, for protopine 7.1 ± 2.3 μM and 0.24 ± 0.021 min^{-1} (**III**: Figures 1 and 2; Table 1).

Irreversibility of inactivation. Bulbocapnine, canadine and protopine displayed irreversible characteristics in the dialysis experiment, similar to those of the positive control compound, isoniazid (**III**: Table 2). The activity of CYP2C19, which had been inactivated by bulbocapnine, canadine, protopine or isoniazid in the presence of the NADPH, was not restored by dialysis or recovered only slightly. Whereas, in the absence of the NADPH in the inactivation mixture, a dramatic recovery of CYP2C19 activity could be achieved. When CYP2C19 was inactivated by tranylcypromine in the presence and absence of the NADPH

in the inactivation mixture, the CYP2C19 activity was fully restored by dialysis in both cases.

Spectroscopic determination of MIC formation. In the cases of bulbocapnine, canadine and protopine, the MIC-associated increase in absorbance was observed in the presence of NADPH, but no MIC-associated increase in absorbance was observed for tranylcypromine. In the absence of NADPH, none of the compounds produced the characteristic peak at ~455 nm (**III**: Figure 3).

These specific *in vitro* criteria were fulfilled to determine that the three isoquinoline alkaloids **bulbocapnine, canadine and protopine are new mechanism-based inactivators of CYP2C19**. These can thus serve as good tools for studying structural and functional elements of CYP2C19 (active site, substrate binding and catalysis). In addition, due to the MBI manner, these alkaloids have the potential to cause clinically relevant drug interactions. Based on these MBI criteria, experimental results plus molecular docking results (**IV**), it is possible that many of the 49 alkaloids examined here have the potential to be mechanism-based inactivators, but further experiments will be needed to verify this proposal. Lately, noscapine have been reported to be a mechanism-based inactivator for CYP2C9 (Zhang et al., 2013) and tetrahydropalmatine for CYPs 1A2, 2D6 and 3A4 (Zhao et al., 2015).

5.6 MOLECULAR DOCKING AND 3D-QSAR STUDIES (IV)

Molecular modeling. Canadine (**20**) (IC_{50} 0.29 μM) was used as the template for the docking analysis due to its rigid molecular structure and potent inhibition. The docking model revealed that the MDP in canadine optimally coordinated towards heme (Figure 17). These alkaloids lie in the hydrophobic pocket formed by residues Ile205, Leu102, Leu233 and Leu237 at the entrance of the active site. Furthermore, a polar interaction was observed between canadine and a hydrophilic residue (Asn107) in close proximity to the active site. For most of the other alkaloids, the hypothetical orientations of docking poses were comparable to that of canadine. The hydrophilic residue Asp293 situated close to the active site of the CYP2C19, can also form a polar interaction with the studied alkaloids.

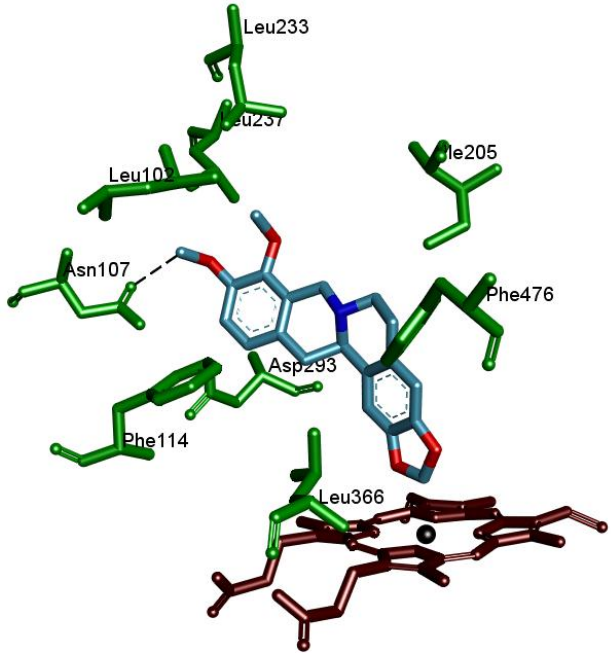


Figure 17. Canadine is surrounded by hydrophobic residues Leu102, Phe114, Ile205, Leu233, Leu237, and Phe476 in the CYP2C19 active site. A polar interaction is formed with Asn107.

The CoMFA analysis was conducted with the PLS algorithm. The optimal number of components in the final model was three, which was obtained using the cross-validation technique. The model displayed good statistical values: $r^2 = 0.94$, standard error of estimate 0.25. The progressive scrambling analysis indicated three components as being optimal based on the minimum value for cSDEP with the maximum q^2 value. The contributions of steric and electrostatic fields of the CoMFA model were 47% and 58%, respectively. The contour maps of the CoMFA models (Figure 18) represent sterically favorable (green contours) and sterically unfavorable regions (yellow contours) as well as electronegative (red contour) and electropositive (blue contour) favorable regions for CYP2C19 inhibition. The sterically favorable and unfavorable regions indicated the optimal volume and shape of the inhibitory compounds. The steric fields suggested that the inhibition potency should increase if a hydrophobic group could occupy these regions. Overall, the steric and electrostatic contributions of the CoMFA are consistent with the shape and properties of the CYP2C19 active site. Corycavamine (**6**) (IC_{50} 0.19 μM), representing a potent MDP derivative, was observed to coordinate towards heme (**IV**: Figure 2b). The molecule consists of two MDP moieties; one of these points to the contour of the sterically favorable region in the hydrophobic pocket near to residues Leu102, Leu205, Leu233 and Leu237.

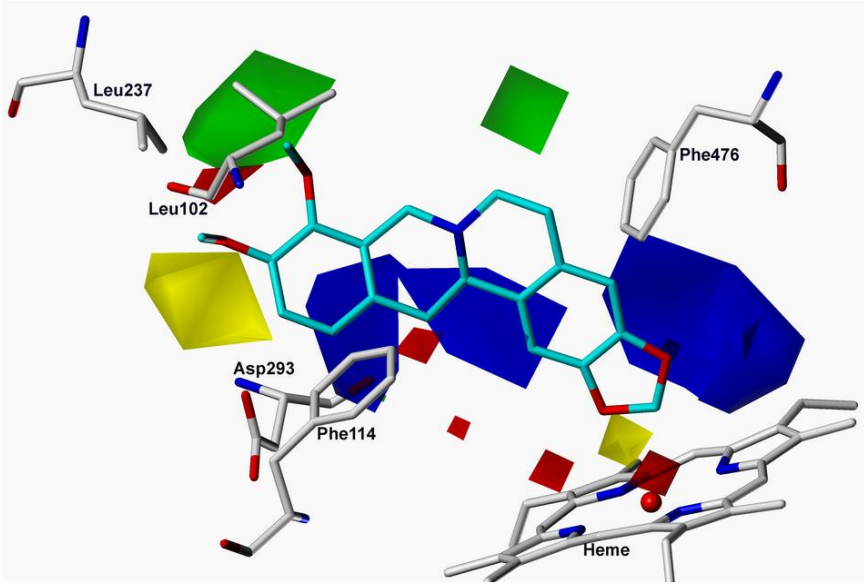


Figure 18. The contour map of the CoMFA model with canadine. The electronegative (red contour) and electropositive (blue contour) favorable regions for inhibition are presented. The steric contours indicate areas where bulky groups increase (green contours) or decrease (yellow contours) inhibitory activity, respectively.

The decisive test for QSAR model robustness is its ability to predict the biological activity of compounds not included in the training set. The predictive ability of the model was evaluated using an external test set of 12 compounds, including alkaloids covering all inhibition potency categories and various structural scaffolds. The correlation between experimental and predicted inhibitory activities was high with an r^2 value of 0.8 (**IV**: Figure 3). The predicted values were within 0.5 log units of the experimental values, with the exception of two compounds (capnoidine **17** and bicuculline **18**). However, the predicted pIC50 values were in agreement with the experimental data within a statistically tolerable error range.

The created CYP2C19 3D-QSAR model can be used to characterize further the molecular properties of CYP2C19 ligands and to predict the CYP2C19 inhibitory activity of new compounds which are structurally close to these isoquinoline alkaloids used to build the model and to identify new inhibitors. Currently known common features of CYP2C19 ligands are lipophilic, aromatic, acidic, neutral or basic molecules with site of oxidation a discrete distance from 2 H-bond acceptor heteroatoms, and typical CYP2C19 substrates are medium-sized basic molecules with 2–3 hydrogen bond acceptors (Raunio et al., 2015). These characteristics are fulfilled for the aromatic and lipophilic, medium-sized and basic molecules in the studied alkaloids. However, 2-3 hydrogen bond acceptors are not found in all alkaloids and some are hydrogen bond donors.

As far as is known, this is the first QSAR study of isoquinoline alkaloids with CYP enzymes. Molecular docking studies of isoquinoline alkaloids with CYP enzymes are also rare. Jiang et al. (2013) have investigated the metabolic site of the isoquinoline alkaloid, corynoline, using molecular docking with CYP3A4. The results showed that the MDP group was closer with heme than other groups in the structure of corynoline, indicating that the MDP group is the most plausible group undergoing CYP3A4-mediated metabolism. Lo et al. (2013) have investigated the interactions of berberine with CYPs 1A1,

1A2 and 1B1 enzymes using molecular docking, as did also Chang et al. (2015). The results showed similar orientations of berberine and its metabolites in the active sites of CYP1 enzymes i.e. the MDP moiety was orientated towards the heme. Thus, their findings are in line with these present results with CYP2C19.

6 Summary and Conclusion

In this work, the inhibitory activity of several plant-derived as well as semisynthetic and synthetic isoquinoline alkaloids was determined towards all of the principal human hepatic drug metabolizing CYP enzymes. The TDI properties of these structurally related isoquinoline alkaloids were evaluated towards CYP2C19 *in vitro*. The alkaloids were docked in the crystal structure of CYP2C19 and 3D-QSAR analysis was performed *in silico*. Several *in vitro* criteria were applied to evaluate whether three representative herbal medicine alkaloids (bulbocapnine, canadine and protopine) were mechanism-based inactivators of CYP2C19. A new fluorometric CYP2C19 progress curve analysis assay was developed to assess CYP2C19 enzyme inactivation kinetics *in vitro*. In addition, a detailed search from a commercial TCM Database was conducted to learn whether the current alkaloids are present in TCM.

The following conclusions can be drawn from the results of the present study:

1. The majority, i.e. 53%, of the studied alkaloids were present in TCM with three of them being used as conventional medicines. Many of these alkaloids occur widely in medicinal plants and at least 33 of the 49 studied alkaloids are likely to be ingested by humans.

2. The current study indicated evidence of a high-affinity interaction between several isoquinoline alkaloids and several human drug-metabolizing CYP enzymes. The most potently inhibited CYP enzyme was CYP3A4, followed by others in rank order: CYP2D6 > CYP2C19 > CYP1A2 > CYP2C9 > CYP2B6 > CYP2C8 > CYP2A6. Only CYP2A6 was not significantly inhibited by any of the alkaloids. It was especially important that many of these alkaloids were potent inhibitors of the clinically important CYP3A4 and CYP2D6 enzymes. Three potent and selective inhibitors for CYP3A4 (corydine, parfumine and 8-methyl-2,3,10,11-tetraethoxyberbine) were identified.

3. The developed CYP2C19 progress curve analysis assay proved to be a valuable approach for screening TDI as well as for identifying test compounds that require more detailed investigations in drug interaction and optimization studies. The approach can also be exploited for determining important kinetic parameters, such as K_I and k_{inact} values. The inactivation kinetic constants (K_I and k_{inact}) for isoniazid and ticlopidine correlated well with published data, confirming the reliability of the method. This CYP2C19 progress curve analysis method represents a novel way of conducting a TDI analysis. This method provides information about CYP inhibitory activity relationships and rank order of early phase compounds in drug discovery and provides biological data for application in molecular modelling studies.

4. Most of the studied alkaloids (42/49) demonstrated TDI of CYP2C19. The presence of the MDP moiety increased the inhibitory potency. Many of the studied alkaloids have the potential to act as mechanism-based inactivators of CYP2C19.

5. Bulbocapnine, canadine and protopine fulfilled *in vitro* MBI criteria of time-dependency, saturation, irreversibility and involvement of the catalysis by CYP2C19. These herbal medicine alkaloids are novel mechanism-based inactivators for CYP2C19. As a precaution concomitant use of e.g. clopidogrel should be discouraged.

6. Molecular modeling and 3D-QSAR analysis showed key interactions between the potent inhibitors with the CYP2C19 active site. The steric and electrostatic field maps in CoMFA were consistent with the crystal structure of CYP2C19. A clear correlation was found between the inhibitor structures and inhibition potency. The CoMFA model constructed was capable of predicting inhibition potency of independent alkaloids. The MDP moiety of most of the alkaloids was coordinated toward the heme.

In conclusion, these results provide a broad perspective of the interactions between novel isoquinoline alkaloids and the most important human CYP enzymes, highlighting their potential to cause clinically meaningful drug interactions. This information should be taken into account in drug discovery and development. This study further confirms that drug metabolism-based interactions can occur between drugs and natural products. Thus, the results highlight the need for scrutinizing the potential interactions between drugs and natural products and herbal preparations. In addition, the benefits of exploiting the developed progress curve analysis method for measuring CYP2C19 catalytic activity (plus other CYPs) in the field of early drug interaction studies should be evaluated in detail. Also spectroscopic determination assay possibilities for screening purposes should be evaluated in detail.

References

Afzelius L, Masimirembwa CM, Karlen A, Andersson TB and Zamora I (2002) Discriminant and quantitative PLS analysis of competitive CYP2C9 inhibitors versus non-inhibitors using alignment independent GRIND descriptors. *J Comput Aided Mol Des* **16**:443-458.

Afzelius L, Zamora I, Ridderstrom M, Andersson TB, Karlen A and Masimirembwa CM (2001) Competitive CYP2C9 inhibitors: enzyme inhibition studies, protein homology modeling, and three-dimensional quantitative structure-activity relationship analysis. *Mol Pharmacol* **59**:909-919.

Alfaro CL (2001) Emerging role of drug interaction studies in drug development: the good, the bad, and the unknown. *Psychopharmacol Bull* **35**:80-93.

Amin AM, Chin LS, Noor DAM, Kader M, Hay YK and Ibrahim B (2017) The Personalization of Clopidogrel Antiplatelet Therapy: The Role of Integrative Pharmacogenetics and Pharmacometabolomics. *Cardiology Research and Practice* 17.

Andersson ML, Bottiger Y, Lindh JD, Wettermark B and Eiermann B (2013) Impact of the drug-drug interaction database SFINX on prevalence of potentially serious drug-drug interactions in primary health care. *Eur J Clin Pharmacol* **69**:565-571.

Aniszewski T (2015) Alkaloids: chemistry, biology, ecology and applications. Elsevier Ltd., Amsterdam.

Backman JT, Kyrklund C, Neuvonen M and Neuvonen PJ (2002) Gemfibrozil greatly increases plasma concentrations of cerivastatin. *Clin Pharmacol Ther* **72**:685-691.

Bapiro TE, Egnell AC, Hasler JA and Masimirembwa CM (2001) Application of higher throughput screening (HTS) inhibition assays to evaluate the interaction of antiparasitic drugs with cytochrome P450s. *Drug Metab Dispos* **29**:30-35.

Barbuti AM and Chen ZS (2015) Paclitaxel Through the Ages of Anticancer Therapy: Exploring Its Role in Chemoresistance and Radiation Therapy. *Cancers (Basel)* **7**:2360-2371.

Baroni M, Costantino G, Cruciani G, Riganelli D, Valigi R and Clementi S (1993) Generating optimal linear PLS eestimations (GOLPE) - An advanced chemometric tool for handling 3D-QSAR problems. *Quantitative Structure-Activity Relationships* **12**:9-20.

Bauman JN, Kelly JM, Tripathy S, Zhao SX, Lam WW, Kalgutkar AS and Obach RS (2009) Can in vitro metabolism-dependent covalent binding data distinguish hepatotoxic from nonhepatotoxic drugs? An analysis using human hepatocytes and liver S-9 fraction. *Chem Res Toxicol* **22**:332-340.

BD Gentest (2000) A high throughput method for measuring cytochrome P450 inhibition. Available at: http://www2.bdbiosciences.com/discovery_labware/gentest/products/HTS_KITS/HTS/hts_summary.shtml

Beaudoin GA and Facchini PJ (2014) Benzylisoquinoline alkaloid biosynthesis in opium poppy. *Planta* **240**:19-32.

Bell L, Bickford S, Nguyen PH, Wang J, He T, Zhang B, Friche Y, Zimmerlin A, Urban L and Bojanic D (2008) Evaluation of fluorescence- and mass spectrometry-based CYP inhibition assays for use in drug discovery. *J Biomol Screen* **13**:343-353.

Berman HM, Battistuz T, Bhat TN, Bluhm WF, Bourne PE, Burkhardt K, Feng Z, Gilliland GL, Iype L, Jain S, Fagan P, Marvin J, Padilla D, Ravichandran V, Schneider B, Thanki N, Weissig H, Westbrook JD and Zardecki C (2002) The Protein Data Bank. *Acta Crystallogr D Biol Crystallogr* **58**:899-907.

Bernhardt R (2006) Cytochromes P450 as versatile biocatalysts. *J Biotechnol* **124**:128-145.

Bertilsson L, Henthorn TK, Sanz E, Tybring G, Sawe J and Villen T (1989) Importance of genetic factors in the regulation of diazepam metabolism: relationship to S-mephenytoin, but not debrisoquin, hydroxylation phenotype. *Clin Pharmacol Ther* **45**:348-355.

Bertomeu-Sanchez JR (2012) Animal experiments, vital forces and courtrooms: Mateu Orfila, Francois Magendie and the study of poisons in nineteenth-century France. *Ann Sci* **69**:1-26.

Betz JM, Fisher KD, Saldanha LG and Coates PM (2007) The NIH analytical methods and reference materials program for dietary supplements. *Anal Bioanal Chem* **389**:19-25.

Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach SR, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J and Wrighton SA (2003) The conduct of in vitro and in vivo drug-drug interaction studies: A PhRMA perspective. *J Clin Pharmacol* **43**:443-469.

Blaisdell J, Mohrenweiser H, Jackson J, Ferguson S, Coulter S, Chanas B, Xi T, Ghanayem B and Goldstein JA (2002) Identification and functional characterization of new potentially defective alleles of human CYP2C19. *Pharmacogenetics* **12**:703-711.

Bode C (2010) The nasty surprise of a complex drug-drug interaction. *Drug Discov Today* **15**:391-395.

Borg JJ, Tanti A, Kouvelas D, Lungu C, Pirozynski M, Serracino-Inglott A and Aislaitner G (2015) European Union pharmacovigilance capabilities: potential for the new legislation. *Ther Adv Drug Saf* **6**:120-140.

Bottiger Y, Laine K, Andersson ML, Korhonen T, Molin B, Ovesjo ML, Tirkkonen T, Rane A, Gustafsson LL and Eiermann B (2009) SFINX-a drug-drug interaction database designed for clinical decision support systems. *Eur J Clin Pharmacol* **65**:627-633.

Boulogne J (2001) [Medical myths and notions in Ancient Greece]. *Med Nowozytna* **8**:33-52.

Breckenridge A (2006) William Withering's legacy--for the good of the patient. *Clin Med (Lond)* **6**:393-397.

Breimer DD and Schellens JH (1990) A 'cocktail' strategy to assess in vivo oxidative drug metabolism in humans. *Trends Pharmacol Sci* **11**:223-225.

Budman DR (1997) Vinorelbine (Navelbine): a third-generation vinca alkaloid. *Cancer Invest* **15**:475-490.

Byrne BE (2003) Drug interactions: a review and update. *Alpha Omegan* **96**:10-17.

Calixto JB (2000) Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Braz J Med Biol Res* **33**:179-189.

Capdevila JH, Holla VR and Falck JR (2005) Cytochrome P450 and the Metabolism and Bioactivation of Arachidonic Acid and Eicosanoids, in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano PR ed) pp 531-551, Kluwer Academic/Plenum Publishers, New York.

Cascorbi I (2012) Drug interactions--principles, examples and clinical consequences. *Dtsch Arztebl Int* **109**:546-555; quiz 556.

Casida JE (1970) Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J Agric Food Chem* **18**:753-772.

Chang YP, Huang CC, Shen CC, Tsai KC and Ueng YF (2015) Differential inhibition of CYP1-catalyzed regioselective hydroxylation of estradiol by berberine and its oxidative metabolites. *Drug Metab Pharmacokinet* **30**:374-383.

Cohen LH, Remley MJ, Raunig D and Vaz AD (2003) In vitro drug interactions of cytochrome p450: an evaluation of fluorogenic to conventional substrates. *Drug Metab Dispos* **31**:1005-1015.

Colombo ML and Bosisio E (1996) Pharmacological activities of Chelidonium majus L (Papaveraceae). *Pharmacol Res* **33**:127-134.

Copeland RA (2005) Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. John Wiley & Sons, Inc., Hoboken, New Jersey.

Correia MA and Ortiz de Montellano PR (2005) Inhibition of Cytochrome P450 Enzymes, in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano PR ed) pp 247-322, Kluwer Academic/Plenum Publishers, New York.

Coutinho Moraes DF, Still DW, Lum MR and Hirsch AM (2015) DNA-Based Authentication of Botanicals and Plant-Derived Dietary Supplements: Where Have We Been and Where Are We Going? *Planta Med* **81**:687-695.

Cragg GM, Grothaus PG and Newman DJ (2014) New horizons for old drugs and drug leads. *J Nat Prod* **77**:703-723.

Cramer RD, Patterson DE and Bunce JD (1988) Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J Am Chem Soc* **110**:5959-5967.

Crespi CL, Miller VP and Penman BW (1997) Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* **248**:188-190.

Crespi CL and Stresser DM (2000) Fluorometric screening for metabolism-based drug-drug interactions. *J Pharmacol Toxicol −ethods* **44**:325-331.

Davis JD, Aarons L and Houston JB (1994) Relationship between enoxacin and ciprofloxacin plasma concentrations and theophylline disposition. *Pharm Res* **11**:1424-1428.

de Graaf C, Vermeulen NPE and Feenstra KA (2005) Cytochrome P450 in silico: An integrative modeling approach. *J Med Chem* **48**:2725-2755.

de Souza Silva JE, Santos Souza CA, da Silva TB, Gomes IA, Brito Gde C, de Souza Araujo AA, de Lyra-Junior DP, da Silva WB and da Silva FA (2014) Use of herbal medicines by elderly patients: A systematic review. *Arch Gerontol Geriatr* **59**:227-233.

De Vos P (2010) European materia medica in historical texts: longevity of a tradition and implications for future use. *J Ethnopharmacol* **132**:28-47.

Dean L (2012) Clopidogrel Therapy and CYP2C19 Genotype, in Medical Genetics Summaries (Pratt V, McLeod H, Dean L, Malheiro A, Rubinstein W ed) pp National Center for Biotechnology Information (US), Bethesda (MD).

Debeljak N, Fink M and Rozman D (2003) Many facets of mammalian lanosterol 14alpha-demethylase from the evolutionarily conserved cytochrome P450 family CYP51. *Arch Biochem Biophys* **409**:159-171.

Denisov IG, Makris TM, Sligar SG and Schlichting I (2005) Structure and chemistry of cytochrome P450. *Chem Rev* **105**:2253-2277.

Desai MC and Chackalamannil S (2008) Rediscovering the role of natural products in drug discovery. *Curr Opin Drug Discov Devel* **11**:436-437.

Desta Z, Zhao X, Shin JG and Flockhart DA (2002) Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin Pharmacokinet* **41**:913-958.

Di L, Kerns EH, Li SQ and Carter GT (2007) Comparison of cytochrome P450 inhibition assays for drug discovery using human liver microsomes with LC-MS, rhCYP450 isozymes with fluorescence, and double cocktail with LC-MS. *Int J Pharm* **335**:1-11.

Di Lorenzo C, Ceschi A, Kupferschmidt H, Lude S, De Souza Nascimento E, Dos Santos A, Colombo F, Frigerio G, Norby K, Plumb J, Finglas P and Restani P (2015) Adverse effects of plant food supplements and botanical preparations: a systematic review with critical evaluation of causality. *Br J Clin Pharmacol* **79**:578-592.

Dickins M, Elcombe CR, Moloney SJ, Netter KJ and Bridges JW (1979) Further studies on the dissociation of the isosafrole metabolite-cytochrome P-450 complex. *Biochem Pharmacol* **28**:231-238.

Dieckhaus CM, Thompson CD, Roller SG and Macdonald TL (2002) Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem Biol Interact* **142**:99-117.

Dominiczak MH (2011) Paracelsus: In Praise of Mavericks. *Clin Chem* **57**:932-934.

Donahue SR, Flockhart DA, Abernethy DR and Ko JW (1997) Ticlopidine inhibition of phenytoin metabolism mediated by potent inhibition of CYP2C19. *Clin Pharmacol Ther* **62**:572-577.

Donato MT, Jimenez N, Castell JV and Gomez-Lechon MJ (2004) Fluorescence-based assays for screening nine cytochrome P450 (P450) activities in intact cells expressing individual human P450 enzymes. *Drug Metab Dispos* **32**:699-706.

Dong D, Wu B, Chow D and Hu M (2012) Substrate selectivity of drug-metabolizing cytochrome P450s predicted from crystal structures and in silico modeling. *Drug Metab Rev* **44**:192-208.

Efferth T and Kaina B (2011) Toxicities by herbal medicines with emphasis to traditional Chinese medicine. *Curr Drug Metab* **12**:989-996.

Efferth T, Zacchino S, Georgiev MI, Liu L, Wagner H and Panossian A (2015) Nobel Prize for artemisinin brings phytotherapy into the spotlight. *Phytomedicine* **22**:A1-3.

EMA (2010) Guideline on the investigation of drug Interactions. EMEA/CHMP/EWP/125211/10. Available at: http://www.emea.europa.eu/htms/human/humanguidelines/efficacy.htm

EMA (2012) Guideline on the Investigation of Drug Interactions. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf

Ervine CM, Matthew DE, Brennan B and Houston JB (1996) Comparison of ketoconazole and fluconazole as cytochrome P450 inhibitors. Use of steady-state infusion approach to achieve plasma concentration-response relationships. *Drug Metab Dispos* **24**:211-215.

Estabrook RW (1996) The remarkable P450s: a historical overview of these versatile hemeprotein catalysts. *FASEB J* **10**:202-204.

Fairman DA, Collins C and Chapple S (2007) Progress curve analysis of CYP1A2 inhibition: A more informative approach to the assessment of mechanism-based inactivation? *Drug Metab Dispos* **35**:2159-2165.

Fang ZZ, Zhang YY, Ge GB, Huo H, Liang SC and Yang L (2010) Time-dependent inhibition (TDI) of CYP3A4 and CYP2C9 by noscapine potentially explains clinical noscapine-warfarin interaction. *Br J Clin Pharmacol* **69**:193-199.

Fang ZZ, Zhang YY, Ge GB, Liang SC, Sun DX, Zhu LL, Dong PP, Cao YF and Yang L (2011) Identification of cytochrome P450 (CYP) isoforms involved in the metabolism of corynoline, and assessment of its herb-drug interactions. *Phytother Res* **25**:256-263.

FDA (2006) Guidance for industry. Drug interaction studies - study design, data analysis, and implications for dosing and labeling. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf

FDA (2012) Guidance for Industry: Drug Interaction Studies – Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf

Fowler MW (2006) Plants, medicines and man. *J Sci Food Agric* **86**:1797-1804.

Fowler S and Zhang HJ (2008) In vitro evaluation of reversible and irreversible cytochrome P450 inhibition: Current status on methodologies and their utility for predicting drug-drug interactions. *Aaps Journal* **10**:410-424.

Franklin MR (1971) The enzymic formation of methylenedioxyphenyl derivative exhibiting an isocyanide-like spectrum with reduced cytochrome P-450 in hepatic microsomes. *Xenobiotica* **1**:581-591.

Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, Shaw DE, Francis P and Shenkin PS (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* **47**:1739-1749.

Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC and Mainz DT (2006) Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem* **49**:6177-6196.

Funayama S and Cordell GA (2015) Alkaloids: a treasury of poisons and medicines. Academic Press, London.

Furst R and Zundorf I (2015) Evidence-Based Phytotherapy in Europe: Where Do We Stand? *Planta Med* **81**:962-967.

Furuta T, Ohashi K, Kamata T, Takashima M, Kosuge K, Kawasaki T, Hanai H, Kubota T, Ishizaki T and Kaneko E (1998) Effect of genetic differences in omeprazole metabolism on cure rates for Helicobacter pylori infection and peptic ulcer. *Ann Intern Med* **129**:1027-1030.

Garcia-Canovas F, Tudela J, Varon R and Vazquez AM (1989) Experimental methods for kinetic study of suicide substrates. *J Enzyme Inhib* **3**:81-90.

Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT and Rostami-Hodjegan A (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Curr Drug Metab* **7**:315-334.

Gilard V, Balayssac S, Tinaugus A, Martins N, Martino R and Malet-Martino M (2015) Detection, identification and quantification by 1H NMR of adulterants in 150 herbal dietary supplements marketed for improving sexual performance. *J Pharm Biomed Anal* **102**:476-493.

Gilca M, Gaman L, Panait E, Stoian I and Atanasiu V (2010) Chelidonium majus--an integrative review: traditional knowledge versus modern findings. *Forsch Komplementmed* **17**:241-248.

Goldstein JA (2001) Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol* **52**:349-355.

Goldstein JA, Faletto MB, Romkessparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM and Ghanayem BI (1994) EVIDENCE THAT CYP2C19 IS THE MAJOR (S)-MEPHENYTOIN 4'-HYDROXYLASE IN HUMANS. *Biochemistry (Mosc)* **33**:1743-1752.

Gomez-Lechon MJ, Lahoz A, Gombau L, Castell JV and Donato MT (2010) In vitro evaluation of potential hepatotoxicity induced by drugs. *Curr Pharm Des* **16**:1963-1977.

Gonzalez FJ, Coughtrie M and Tukey RH (2011) Drug metabolism, in Goodman & Gilmans's The Pharmacological Basis of Therapeutics (Brunton LL, Chabner B, Knollman B ed) pp 123-143, McGraw-Hill, New York.

Goodford PJ (1985) A computational-procedure for determining energetically favorable binding-sites on biologically important macromolecules. *J Med Chem* **28**:849-857.

Gotoh O (1992) Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J Biol Chem* **267**:83-90.

Gotti R (2011) Capillary electrophoresis of phytochemical substances in herbal drugs and medicinal plants. *J Pharm Biomed Anal* **55**:775-801.

Grime KH, Bird J, Ferguson D and Riley RJ (2009) Mechanism-based inhibition of cytochrome P450 enzymes: An evaluation of early decision making in vitro approaches and drug-drug interaction prediction methods. *Eur J Pharm Sci* **36**:175-191.

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KHJ, Lu C, Nomeir AA, Seibert E, Skordos KW, Tonn GR, Van Horn R, Wang RW, Wong YN, Yang TJ and Obach RS (2009) The Conduct of in Vitro Studies to Address Time-Dependent Inhibition of Drug-Metabolizing Enzymes: A Perspective of the Pharmaceutical Research and Manufacturers of America. *Drug Metab Dispos* **37**:1355-1370.

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* **14**:611-650.

Guengerich FP (2003) Cytochromes P450, drugs, and diseases. *Mol Interv* **3**:194-204.

Guengerich FP (2008) Cytochrome P450 and chemical toxicology. *Chem Res Toxicol* **21**:70-83.

Guengerich FP (2013) Kinetic deuterium isotope effects in cytochrome P450 oxidation reactions. *J Labelled Comp Radiopharm* **56**:428-431.

Hagel JM and Facchini PJ (2013) Benzylisoquinoline alkaloid metabolism: a century of discovery and a brave new world. *Plant Cell Physiol* **54**:647-672.

Hajar R (2012) The air of history: early medicine to galen (part I). *Heart Views* **13**:120-128.

Halgren TA, Murphy RB, Friesner RA, Beard HS, Frye LL, Pollard WT and Banks JL (2004) Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J Med Chem* **47**:1750-1759.

Hardwick JP (2008) Cytochrome P450 omega hydroxylase (CYP4) function in fatty acid metabolism and metabolic diseases. *Biochem Pharmacol* **75**:2263-2275.

Harvey AL (2008) Natural products in drug discovery. *Drug Discov Today* **13**:894-901.

He SM, Chan E and Zhou SF (2011) ADME properties of herbal medicines in humans: evidence, challenges and strategies. *Curr Pharm Des* **17**:357-407.

Heikkila T, Lekander T and Raunio H (2006) Use of an online surveillance system for screening drug interactions in prescriptions in community pharmacies. *Eur J Clin Pharmacol* **62**:661-665.

Hermann R and von Richter O (2012) Clinical evidence of herbal drugs as perpetrators of pharmacokinetic drug interactions. *Planta Med* **78**:1458-1477.

Hines RN and Prough RA (1980) The characterization of a inhibitory complex formed with cytochrome P-450 and a metabolite of 1,1-disubstituted hydrazines. *J Pharmacol Exp Ther* **214**:80-86.

Hodgson E, Roe RM, Mailman RB and Chambers JE (2015) Dictionary of Toxicology. Academic Press, UK, USA.

Huang SM, Strong JM, Zhang L, Reynolds KS, Nallani S, Temple R, Abraham S, Habet SA, Baweja RK, Burckart GJ, Chung S, Colangelo P, Frucht D, Green MD, Hepp P, Karnaukhova E, Ko HS, Lee JI, Marroum PJ, Norden JM, Qiu W, Rahman A, Sobel S, Stifano T, Thummel K, Wei XX, Yasuda S, Zheng JH, Zhao H and Lesko LJ (2008) New era in drug interaction evaluation: US Food and Drug

Administration update on CYP enzymes, transporters, and the guidance process. *J Clin Pharmacol* **48**:662-670.

Höltje HD, Sippl W, Rognan D and Folkers G (2003) Molecular Modeling: Basic Principles and Applications. WILEY-VCH GmbH & Co. KGaA, Weinheim.

Ingelman-Sundberg M, Sim SC, Gomez A and Rodriguez-Antona C (2007) Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol Ther* **116**:496-526.

Ito K, Brown HS and Houston JB (2004) Database analyses for the prediction of in vivo drug-drug interactions from in vitro data. *Br J Clin Pharmacol* **57**:473-486.

Iyanagi T (2007) Molecular mechanism of phase I and phase II drug-metabolizing enzymes: implications for detoxification. *Int Rev Cytol* **260**:35-112.

Jeetu G and Anusha G (2010) Pharmacovigilance: a worldwide master key for drug safety monitoring. *J Young Pharm* **2**:315-320.

Ji HF, Li XJ and Zhang HY (2009) Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia? *EMBO Rep* **10**:194-200.

Jiang F, Jiang AF, Zhang LG, Li YZ and Yu SE (2013) Molecular Docking to Predict the Metabolic Site of Corynoline and the Possible Drug-Drug Interaction. *Latin American Journal of Pharmacy* **32**:1581-1583.

Johnson EF, Connick JP, Reed JR, Backes WL, Desai MC, Xu L, Estrada DF, Laurence JS and Scott EE (2014) Correlating structure and function of drug-metabolizing enzymes: progress and ongoing challenges. *Drug Metab Dispos* **42**:9-22.

Kalgutkar AS, Obach RS and Maurer TS (2007) Mechanism-based inactivation of cytochrome P450 enzymes: Chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. *Curr Drug Metab* **8**:407-447.

Kamel A and Harriman S (2013) Inhibition of cytochrome P450 enzymes and biochemical aspects of mechanism-based inactivation (MBI). *Drug Discov Today Technol* **10**:e177-189.

Kapitulnik J, Pelkonen O, Gundert-Remy U, Dahl SG and Boobis AR (2009) Effects of pharmaceuticals and other active chemicals at biological targets: mechanisms, interactions, and integration into PB-PK/PD models. *Expert Opinion on Therapeutic Targets* **13**:867-887.

Kauppinen H, Ahonen R and Timonen J (2017) The impact of electronic prescriptions on medication safety in Finnish community pharmacies: A survey of pharmacists. *Int J Med Inf* **100**:56-62.

Kell DB (2013) Finding novel pharmaceuticals in the systems biology era using multiple effective drug targets, phenotypic screening and knowledge of transporters: where drug discovery went wrong and how to fix it. *Febs j* **280**:5957-5980.

Kelly LE and Madadi P (2012) Is there a role for therapeutic drug monitoring with codeine? *Ther Drug Monit* **34**:249-256.

Kennedy J, Wang CC and Wu CH (2008) Patient Disclosure about Herb and Supplement Use among Adults in the US. *Evid Based Complement Alternat Med* **5**:451-456.

Kirchmair J, Williamson MJ, Tyzack JD, Tan L, Bond PJ, Bender A and Glen RC (2012) Computational prediction of metabolism: sites, products, SAR, P450 enzyme dynamics, and mechanisms. *J Chem Inf Model* **52**:617-648.

Klebe G, Abraham U and Mietzner T (1994) Molecular similarity indexes in a comparative-analysis (COMSIA) of drug molecules to correlate and predict their biological activity. *J Med Chem* **37**:4130-4146.

Klingenberg M (1958) Pigments of rat liver microsomes. *Arch Biochem Biophys* **75**:376-386.

Knapp S, Lamas G, Lughadha EN and Novarino G (2004) Stability or stasis in the names of organisms: the evolving codes of nomenclature. *Philos Trans R Soc Lond B Biol Sci* **359**:611-622.

Korhonen LE, Rahnasto M, Mahonen NJ, Wittekindt C, Poso A, Juvonen RO and Raunio H (2005) Predictive three-dimensional quantitative structure-activity relationship of cytochrome P450 1A2 inhibitors. *J Med Chem* **48**:3808-3815.

Korhonen LE, Turpeinen M, Rahnasto M, Wittekindt C, Poso A, Pelkonen O, Raunio H and Juvonen RO (2007) New potent and selective cytochrome P450 2B6 (CYP2B6) inhibitors based on three-dimensional quantitative structure-activity relationship (3D-QSAR) analysis. *Br J Pharmacol* **150**:932-942.

Kramer MA and Tracy TS (2012) Enzyme Kinetics of Drug-Metabolizing Reactions and Drug-Drug Interactions, in Encyclopedia of Drug Metabolism and Interactions (Lyubimov A ed) pp 75-99, John Wiley & Sons, New Jersey.

Kumar R and Gupta D (2016) Identification of CYP1B1-specific candidate inhibitors using combination of in silico screening, integrated knowledge-based filtering, and molecular dynamics simulations. *Chem Biol Drug Des* **88**:730-739.

Lahoz A, Donato MT, Castell JV and Gomez-Lechon MJ (2008) Strategies to in vitro assessment of major human CYP enzyme activities by using liquid chromatography tandem mass spectrometry. *Curr Drug Metab* **9**:12-19.

Lee SJ (2012) Clinical Application of CYP2C19 Pharmacogenetics Toward More Personalized Medicine. *Front Genet* **3**:318.

Leite PM, Martins MA and Castilho RO (2016) Review on mechanisms and interactions in concomitant use of herbs and warfarin therapy. *Biomed Pharmacother* **83**:14-21.

Lewis DF and Ito Y (2009) Human P450s involved in drug metabolism and the use of structural modelling for understanding substrate selectivity and binding affinity. *Xenobiotica* **39**:625-635.

Lewis DFV (2004) 57 varieties: the human cytochromes P450. *Pharmacogenomics* **5**:305-318.

Li JW and Vederas JC (2009) Drug discovery and natural products: end of an era or an endless frontier? *Science* **325**:161-165.

Lin JH and Lu AY (1998) Inhibition and induction of cytochrome P450 and the clinical implications. *Clin Pharmacokinet* **35**:361-390.

Lin JH and Lu AY (2001) Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu Rev Pharmacol Toxicol* **41**:535-567.

Lin T, Pan K, Mordenti J and Pan L (2007) In vitro assessment of cytochrome P450 inhibition: strategies for increasing LC/MS-based assay throughput using a one-point IC(50) method and multiplexing high-performance liquid chromatography. *J Pharm Sci* **96**:2485-2493.

Lo SN, Chang YP, Tsai KC, Chang CY, Wu TS and Ueng YF (2013) Inhibition of CYP1 by berberine, palmatine, and jatrorrhizine: selectivity, kinetic characterization, and molecular modeling. *Toxicol Appl Pharmacol* **272**:671-680.

Lu P, Schrag ML, Slaughter DE, Raab CE, Shou M and Rodrigues AD (2003) Mechanism-based inhibition of human liver microsomal cytochrome P450 1A2 by zileuton, a 5-lipoxygenase inhibitor. *Drug Metab Dispos* **31**:1352-1360.

Makris TM, Denisov I, Schlichting I and Sligar SG (2005) Activation of Molecular Oxygen by Cytochrome P450, in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano PR ed) pp 149-182, Kluwer Academic/Plenum Publishers, New York.

Manohar N, Adam TJ, Pakhomov SV, Melton GB and Zhang R (2015) Evaluation of Herbal and Dietary Supplement Resource Term Coverage. *Stud Health Technol Inform* **216**:785-789.

Mao B, Gozalbes R, Barbosa F, Migeon J, Merrick S, Kamm K, Wong E, Costales C, Shi W, Wu C and Froloff N (2006) QSAR modeling of in vitro inhibition of cytochrome P450 3A4. *J Chem Inf Model* **46**:2125-2134.

Mao X, Peng Y and Zheng J (2015) In Vitro and In Vivo Characterization of Reactive Intermediates of Corynoline. *Drug Metab Dispos* **43**:1491-1498.

Mayhew BS, Jones DR and Hall SD (2000) An in vitro model for predicting in vivo inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metab Dispos* **28**:1031-1037.

McEneny-King A, Osman W, Edginton AN and Rao PP (2017) Cytochrome P450 binding studies of novel tacrine derivatives: Predicting the risk of hepatotoxicity. *Bioorg Med Chem Lett*

Meyer A and Imming P (2011) Benzylisoquinoline Alkaloids from the Papaveraceae: The Heritage of Johannes Gadamer (1867-1928). *J Nat Prod* **74**:2482-2487.

Meyer UA (2004) Pharmacogenetics - five decades of therapeutic lessons from genetic diversity. *Nat Rev Genet* **5**:669-676.

Meyer UA and Zanger UM (1997) Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annu Rev Pharmacol Toxicol* **37**:269-296.

Miller VP, Stresser D, Crespi CL and Charles L (2001) The use of fluorescein aryl ethers in high throughput cytochrome P450 inhibition assays. PCT Int. Appl. Patent No. WO 2001014361 A1

Mishra BB and Tiwari VK (2011) Natural products: an evolving role in future drug discovery. *Eur J Med Chem* **46**:4769-4807.

Mo SL, Liu WF, Chen Y, Luo HB, Sun LB, Chen XW, Zhou ZW, Sneed KB, Li CG, Du YM, Liang J and Zhou SF (2012) Ligand- and protein-based modeling studies of the inhibitors of human

cytochrome P450 2D6 and a virtual screening for potential inhibitors from the Chinese herbal medicine, Scutellaria baicalensis (Huangqin,Baikal Skullcap). *Comb Chem High Throughput Screen* **15**:36-80.

Moody GC, Griffin SJ, Mather AN, McGinnity DF and Riley RJ (1999) Fully automated analysis of activities catalysed by the major human liver cytochrome P450 (CYP) enzymes: assessment of human CYP inhibition potential. *Xenobiotica* **29**:53-75.

Morissette P, Hreiche R and Turgeon J (2005) Drug-induced long QT syndrome and torsade de pointes. *Can J Cardiol* **21**:857-864.

Muakkassah SF, Bidlack WR and Yang WCT (1981) Mechanism of the inhibitory action of isoniazid on microsomal drug metabolism. *Biochem Pharmacol* **30**:1651-1658.

Nakajima M, Suzuki M, Yamaji R, Takashina H, Shimada N, Yamazaki H and Yokoi T (1999) Isoform selective inhibition and inactivation of human cytochrome P450s by methylenedioxyphenyl compounds. *Xenobiotica* **29**:1191-1202.

Nakamura K, Goto F, Ray WA, McAllister CB, Jacqz E, Wilkinson GR and Branch RA (1985) Interethnic differences in genetic polymorphism of debrisoquin and mephenytoin hydroxylation between Japanese and Caucasian populations. *Clin Pharmacol Ther* **38**:402-408.

Narashimamurthy J, Rao AR and Sastry GN (2004) Aromatase inhibitors: a new paradigm in breast cancer treatment. *Curr Med Chem Anticancer Agents* **4**:523-534.

Naritomi Y, Teramura Y, Terashita S and Kagayama A (2004) Utility of microtiter plate assays for human cytochrome P450 inhibition studies in drug discovery: application of simple method for detecting quasi-irreversible and irreversible inhibitors. *Drug Metab Pharmacokinet* **19**:55-61.

Nawrot R, Zauber H and Schulze WX (2014) Global proteomic analysis of Chelidonium majus and Corydalis cava (Papaveraceae) extracts revealed similar defense-related protein compositions. *Fitoterapia* **94**:77-87.

Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levin W and et al. (1987) The P450 gene superfamily: recommended nomenclature. *DNA* **6**:1-11.

Nebert DW and Russell DW (2002) Clinical importance of the cytochromes P450. *Lancet* **360**:1155-1162.

Nelson DR (2006) Cytochrome P450 nomenclature, 2004. *Methods Mol Biol* **320**:1-10.

Newman DJ and Cragg GM (2007) Natural products as sources of new drugs over the last 25 years. *J Nat Prod* **70**:461-477.

Newman DJ and Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod* **75**:311-335.

Newman DJ and Cragg GM (2016) Natural Products as Sources of New Drugs from 1981 to 2014. *J Nat Prod* **79**:629-661.

Newman DJ, Cragg GM and Snader KM (2000) The influence of natural products upon drug discovery. *Nat Prod Rep* **17**:215-234.

NiceData Software (2005) Traditional Chinese Medicines Database. Version 2.1, CambridgeSoft Corporation, Cambridge, MA, USA.

Nishimura Y, Kurata N, Iwase M and Yasuhara H (2003) Mechanism-based inactivation of human CYP1A2 and CYP2C19 mediated metabolism by isoniazid. *Drug Metab Rev* **35**:100.

Nishiya Y, Hagihara K, Kurihara A, Okudaira N, Farid NA, Okazaki O and Ikeda T (2009) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39**:836-843.

Obach RS (2009) Predicting drug-drug interactions from in vitro drug metabolism data: challenges and recent advances. *Current Opinion in Drug Discovery & Development* **12**:81-89.

Obach RS, Walsky RL, Venkatakrishnan K, Gaman EA, Houston JB and Tremaine LM (2006) The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J Pharmacol Exp Ther* **316**:336-348.

Obach RS, Walsky RL, Venkatakrishnan K, Houston JB and Tremaine LM (2005) In vitro cytochrome P450 inhibition data and the prediction of drug-drug interactions: qualitative relationships, quantitative predictions, and the rank-order approach. *Clin Pharmacol Ther* **78**:582-592.

Ohlsson S, Holm L, Myrberg O, Sundstrom A and Yue QY (2008) Noscapine may increase the effect of warfarin. *Br J Clin Pharmacol* **65**:277-278.

Olkkola KT, Backman JT and Neuvonen PJ (1994) Midazolam should be avoided in patients receiving the systemic antimycotics ketoconazole or itraconazole. *Clin Pharmacol Ther* **55**:481-485.

Olsen L, Oostenbrink C and Jorgensen FS (2015) Prediction of cytochrome P450 mediated metabolism. *Adv Drug Deliv Rev* **86**:61-71.

Omura T and Sato R (1962) A new cytochrome in liver microsomes. *J Biol Chem* **237**:1375-1376.

Onakpoya IJ, Heneghan CJ and Aronson JK (2015) Delays in the post-marketing withdrawal of drugs to which deaths have been attributed: a systematic investigation and analysis. *BMC Med* **13**:26.

Oprea TI and Garcia AE (1996) Three-dimensional quantitative structure-activity relationships of steroid aromatase inhibitors. *J Comput Aided Mol Des* **10**:186-200.

Orr ST, Ripp SL, Ballard TE, Henderson JL, Scott DO, Obach RS, Sun H and Kalgutkar AS (2012) Mechanism-based inactivation (MBI) of cytochrome P450 enzymes: structure-activity relationships and discovery strategies to mitigate drug-drug interaction risks. *J Med Chem* **55**:4896-4933.

Orr STM, Ripp SL, Ballard TE, Henderson JL, Scott DO, Obach RS, Sun H and Kalgutkar AS (2012) Mechanism-Based Inactivation (MBI) of Cytochrome P450 Enzymes: Structure-Activity Relationships and Discovery Strategies To Mitigate Drug-Drug Interaction Risks. *J Med Chem* **55**:4896-4933.

Ortiz de Montellano PR (2012) Structure and Function of Cytochrome P450 Enzymes, in Encyclopedia of Drug Metabolism and Interactions, Volume 1, Fundamentals and Enzyme Systems (Lyubimov AV ed) pp 161-179, John Wiley & Sons, Inc, New Jersey.

Ortiz de Montellano PR and De Voss JJ (2005) Substrate Oxidation by Cytochrome P450 Enzymes, in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano PR ed) pp 183-245, Kluwer Academic/Plenum Publishers, New York.

Pan Y, Chen W, Xu Y, Yi X, Han Y, Yang Q, Li X, Huang L, Johnston SC, Zhao X, Liu L, Zhang Q, Wang G, Wang Y and Wang Y (2017) Genetic Polymorphisms and Clopidogrel Efficacy for Acute Ischemic Stroke or Transient Ischemic Attack: A Systematic Review and Meta-Analysis. *Circulation* **135**:21-33.

Pelkonen O (2015) Drug Metabolism - From In Vitro to In Vivo, From Simple to Complex: Reflections of the BCPT Nordic Prize 2014 Awardee. *Basic Clin Pharmacol Toxicol* **117**:147-155.

Pelkonen O, Maenpaa J, Taavitsainen P, Rautio A and Raunio H (1998) Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* **28**:1203-1253.

Pelkonen O, Pasanen M, Tolonen A, Koskinen M, Hakkola J, Abass K, Laine J, Hakkinen M, Juvonen R, Auriola S, Storvik M, Huuskonen P, Rousu T and Rahikkala M (2015) Reactive metabolites in early drug development: predictive in vitro tools. *Curr Med Chem* **22**:538-550.

Pelkonen O and Raunio H (2005) In vitro screening of drug metabolism during drug development: can we trust the predictions? *Expert Opin Drug Metab Toxicol* **1**:49-59.

Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J and Raunio H (2008) Inhibition and induction of human cytochrome P450 enzymes: current status. *Arch Toxicol* **82**:667-715.

Pelkonen O, Turpeinen M and Raunio H (2011) In vivo-in vitro-in silico pharmacokinetic modelling in drug development: current status and future directions. *Clin Pharmacokinet* **50**:483-491.

Pelkonen O, Xu Q and Fan TP (2014) Why is Research on Herbal Medicinal Products Important and How Can We Improve Its Quality? *J Tradit Complement Med* **4**:1-7.

Perera R, Sono M, Sigman JA, Pfister TD, Lu Y and Dawson JH (2003) Neutral thiol as a proximal ligand to ferrous heme iron: implications for heme proteins that lose cysteine thiolate ligation on reduction. *Proc Natl Acad Sci U S A* **100**:3641-3646.

Poirier D, Roy J, Cortes-Benitez F and Dutour R (2016) Targeting cytochrome P450 (CYP) 1B1 with steroid derivatives. *Bioorg Med Chem Lett* **26**:5272-5276.

Polasek TM, Elliot DJ, Somogyi AA, Gillam EM, Lewis BC and Miners JO (2006) An evaluation of potential mechanism-based inactivation of human drug metabolizing cytochromes P450 by monoamine oxidase inhibitors, including isoniazid. *Br J Clin Pharmacol* **61**:570-584.

Polasek TM and Miners JO (2007) In vitro approaches to investigate mechanism-based inactivation of CYP enzymes. *Expert Opin Drug Metab Toxicol* **3**:321-329.

Poso A, Gynther J and Juvonen R (2001) A comparative molecular field analysis of cytochrome P450 2A5 and 2A6 inhibitors. *J Comput Aided Mol Des* **15**:195-202.

Poulos TL, Finzel BC, Gunsalus IC, Wagner GC and Kraut J (1985) The 2.6-A crystal structure of Pseudomonas putida cytochrome P-450. *J Biol Chem* **260**:16122-16130.

Poulos TL and Johnson EF (2005) Structures of Cytochrome P450 Enzymes, in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano PR ed) pp 87-114, Kluwer Academic/Plenum Publishers, New York.

Prueksaritanont T, Chu X, Gibson C, Cui D, Yee KL, Ballard J, Cabalu T and Hochman J (2013) Drug-drug interaction studies: regulatory guidance and an industry perspective. *Aaps j* **15**:629-645.

Qi XY, Liang SC, Ge GB, Liu Y, Dong PP, Zhang JW, Wang AX, Hou J, Zhu LL, Yang L and Tu CX (2013) Inhibitory effects of sanguinarine on human liver cytochrome P450 enzymes. *Food Chem Toxicol* **56**:392-397.

Rahnasto M, Raunio H, Poso A, Wittekindt C and Juvonen RO (2005) Quantitative structure-activity relationship analysis of inhibitors of the nicotine metabolizing CYP2A6 enzyme. *J Med Chem* **48**:440-449.

Rahnasto M, Wittekindt C, Juvonen RO, Turpeinen M, Petsalo A, Pelkonen O, Poso A, Stahl G, Holtje HD and Raunio H (2008) Identification of inhibitors of the nicotine metabolising CYP2A6 enzyme--an in silico approach. *Pharmacogenomics J* **8**:328-338.

Ramesh M and Bharatam PV (2014) Importance of hydrophobic parameters in identifying appropriate pose of CYP substrates in cytochromes. *Eur J Med Chem* **71**:15-23.

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I and Fridlender B (2002) Plants and human health in the twenty-first century. *Trends Biotechnol* **20**:522-531.

Rasool S and Mohamed R (2015) Plant cytochrome P450s: nomenclature and involvement in natural product biosynthesis. *Protoplasma*

Raunio H, Kuusisto M, Juvonen RO and Pentikainen OT (2015) Modeling of interactions between xenobiotics and cytochrome P450 (CYP) enzymes. *Front Pharmacol* **6**:123.

Reynald RL, Sansen S, Stout CD and Johnson EF (2012) Structural characterization of human cytochrome P450 2C19: active site differences between P450s 2C8, 2C9, and 2C19. *J Biol Chem* **287**:44581-44591.

Richter T, Murdter TE, Heinkele G, Pleiss J, Tatzel S, Schwab M, Eichelbaum M and Zanger UM (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J Pharmacol Exp Ther* **308**:189-197.

Riley RJ, Grime K and Weaver R (2007) Time-dependent CYP inhibition. *Expert Opin Drug Metab Toxicol* **3**:51-66.

Riley RJ and Wilson CE (2015) Cytochrome P450 time-dependent inhibition and induction: advances in assays, risk analysis and modelling. *Expert Opin Drug Metab Toxicol* **11**:557-572.

Roe AL, Paine MF, Gurley BJ, Brouwer KR, Jordan S and Griffiths JC (2016) Assessing Natural Product-Drug Interactions: An End-to-End Safety Framework. *Regul Toxicol Pharmacol* **76**:1-6.

Rosenborg S, Stenberg M, Otto S, Ostervall J, Masquelier M, Yue QY, Bertilsson L and Eliasson E (2010) Clinically Significant CYP2C Inhibition by Noscapine but Not by Glucosamine. *Clin Pharmacol Ther* **88**:343-346.

Rossner S (2006) Carl von Linne (1707-1778) - Stephan's corner. *Obesity Reviews* **7**:371-371.

Rowland P, Blaney FE, Smyth MG, Jones JJ, Leydon VR, Oxbrow AK, Lewis CJ, Tennant MG, Modi S, Eggleston DS, Chenery RJ and Bridges AM (2006) Crystal structure of human cytochrome P450 2D6. *J Biol Chem* **281**:7614-7622.

Sahoo N, Manchikanti P and Dey S (2010) Herbal drugs: standards and regulation. *Fitoterapia* **81**:462-471.

Salsali M, Holt A and Baker GB (2004) Inhibitory effects of the monoamine oxidase inhibitor tranylcypromine on the cytochrome P450 enzymes CYP2C19, CYP2C9, and CYP2D6. *Cell Mol Neurobiol* **24**:63-76.

Sansen S, Yano JK, Reynald RL, Schoch GA, Griffin KJ, Stout CD and Johnson EF (2007) Adaptations for the oxidation of polycyclic aromatic hydrocarbons exhibited by the structure of human P450 1A2. *J Biol Chem* **282**:14348-14355.

Schenkman JB, Sligar SG and Cinti DL (1982) Substrate interaction with cytochrome P450, in Hepatic cytochrome P-450 monooxygenase system (Schenkman JB, Kupfer D ed) pp 587-615, Pergamon Press, New York.

Schrödinger (2011) LigPrep, version 2.5, Schrödinger, LLC, New York, NY.

Schrödinger (2012a) Prime, version 3.0, Schrödinger, LLC, New York, NY.

Schrödinger (2012b) Glide, versions 3.0 and 5.8, Schrödinger, LLC, New York, NY.

Schrödinger (2013) Maestro, version 9.6, Schrödinger, LLC, New York, NY.

Scordo MG, Melhus H, Stjernberg E, Edvardsson AM and Wadelius M (2008) Warfarin-noscapine interaction: a series of four case reports. *Ann Pharmacother* **42**:448-450.

Scott SA, Owusu Obeng A and Hulot JS (2014) Antiplatelet drug interactions with proton pump inhibitors. *Expert Opin Drug Metab Toxicol* **10**:175-189.

Sevior DK, Hokkanen J, Tolonen A, Abass K, Tursas L, Pelkonen O and Ahokas JT (2010) Rapid screening of commercially available herbal products for the inhibition of major human hepatic cytochrome P450 enzymes using the N-in-one cocktail. *Xenobiotica* **40**:245-254.

Sevrioukova IF and Poulos TL (2017) Structural basis for regiospecific midazolam oxidation by human cytochrome P450 3A4. *Proc Natl Acad Sci U S A* **114**:486-491.

Shen B (2015) A New Golden Age of Natural Products Drug Discovery. *Cell* **163**:1297-1300.

Shi S and Klotz U (2012) Drug interactions with herbal medicines. *Clin Pharmacokinet* **51**:77-104.

Shityakov S, Puskas I, Roewer N, Forster C and Broscheit J (2014) Three-dimensional quantitative structure-activity relationship and docking studies in a series of anthocyanin derivatives as cytochrome P450 3A4 inhibitors. *Adv Appl Bioinform Chem* **7**:11-21.

Silverman RB (1995) Mechanism-based enzyme inactivators. *Methods Enzymol* **249**:240-283.

Simpson ER (2000) Role of aromatase in sex steroid action. *J Mol Endocrinol* **25**:149-156.

Singh D, Kashyap A, Pandey RV and Saini KS (2011) Novel advances in cytochrome P450 research. *Drug Discov Today* **16**:793-799.

Singla D, Sharma A, Kaur J, Panwar B and Raghava GP (2010) BIAdb: a curated database of benzylisoquinoline alkaloids. *BMC Pharmacol* **10**:4.

Sohn DR, Kwon JT, Kim HK and Ishizaki T (1997) Metabolic disposition of lansoprazole in relation to the S-mephenytoin 4'-hydroxylation phenotype status. *Clin Pharmacol Ther* **61**:574-582.

Sridhar J, Liu J, Foroozesh M and Stevens CL (2012) Insights on cytochrome p450 enzymes and inhibitors obtained through QSAR studies. *Molecules* **17**:9283-9305.

Stevigny C, Bailly C and Quetin-Leclercq J (2005) Cytotoxic and antitumor potentialities of aporphinoid alkaloids. *Curr Med Chem Anticancer Agents* **5**:173-182.

Stickel F, Egerer G and Seitz HK (2000) Hepatotoxicity of botanicals. *Public Health Nutr* **3**:113-124.

Stickel F and Shouval D (2015) Hepatotoxicity of herbal and dietary supplements: an update. *Arch Toxicol* **89**:851-865.

Storbeck KH, Swart P and Swart AC (2007) Cytochrome P450 side-chain cleavage: Insights gained from homology modeling. *Mol Cell Endocrinol* **265**:65-70.

Stresser DM, Mao J, Kenny JR, Jones BC and Grime K (2014) Exploring concepts of in vitro time-dependent CYP inhibition assays. *Expert Opin Drug Metab Toxicol* **10**:157-174.

Stresser DM, Turner SD, Blanchard AP, Miller VP and Crespi CL (2002) Cytochrome P450 fluorometric substrates: identification of isoform-selective probes for rat CYP2D2 and human CYP3A4. *Drug Metab Dispos* **30**:845-852.

Suvarna V (2010) Phase IV of Drug Development. *Perspect Clin Res* **1**:57-60.

Tamblyn R, Huang A, Perreault R, Jacques A, Roy D, Hanley J, McLeod P and Laprise R (2003) The medical office of the 21st century (MOXXI): effectiveness of computerized decision-making support in reducing inappropriate prescribing in primary care. *CMAJ* **169**:549-556.

Tateishi T, Kumai T, Watanabe M, Nakura H, Tanaka M and Kobayashi S (1999) Ticlopidine decreases the in vivo activity of CYP2C19 as measured by omeprazole metabolism. *Br J Clin Pharmacol* **47**:454-457.

Tennant M and McRee DE (2001) The first structure of a microsomal P450--implications for drug discovery. *Curr Opin Drug Discov Devel* **4**:671-677.

Testa B, Pedretti A and Vistoli G (2012) Reactions and enzymes in the metabolism of drugs and other xenobiotics. *Drug Discov Today* **17**:549-560.

Thompson RA, Isin EM, Ogese MO, Mettetal JT and Williams DP (2016) Reactive Metabolites: Current and Emerging Risk and Hazard Assessments. *Chem Res Toxicol* **29**:505-533.

Toivo TM, Mikkola JAV, Laine K and Airaksinen M (2016) Identifying high risk medications causing potential drug-drug interactions in outpatients: A prescription database study based on an online surveillance system. *Research in Social & Administrative Pharmacy* **12**:559-568.

Tripos International (2011) SYBYL-X 2.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.

Tucker GT, Houston JB and Huang SM (2001) Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential--towards a consensus. *Br J Clin Pharmacol* **52**:107-117.

Turpeinen M, Korhonen LE, Tolonen A, Uusitalo J, Juuonen R, Raunio H and Pelkonen A (2006) Cytochrome P450 (CYP) inhibition screening: Comparison of three tests. *Eur J Pharm Sci* **29**:130-138.

Ullrich V and Schnabel KH (1973) Formation and binding of carbanions by cytochrome P-450 of liver microsomes. *Drug Metab Dispos* **1**:176-183.

Unwalla RJ, Cross JB, Salaniwal S, Shilling AD, Leung L, Kao J and Humblet C (2010) Using a homology model of cytochrome P450 2D6 to predict substrate site of metabolism. *J Comput Aided Mol Des* **24**:237-256.

Waley SG (1980) Kinetics of suicide substrates. *Biochem J* **185**:771-773.

Walsky RL and Obach RS (2004) Validated assays for human cytochrome P450 activities. *Drug Metab Dispos* **32**:647-660.

Wang Y, Xing J, Xu Y, Zhou N, Peng J, Xiong Z, Liu X, Luo X, Luo C, Chen K, Zheng M and Jiang H (2015) In silico ADME/T modelling for rational drug design. *Q Rev Biophys* **48**:488-515.

Wani MC and Horwitz SB (2014) Nature as a remarkable chemist: a personal story of the discovery and development of Taxol. *Anticancer Drugs* **25**:482-487.

Varma MV, Pang KS, Isoherranen N and Zhao P (2015) Dealing with the complex drug-drug interactions: towards mechanistic models. *Biopharm Drug Dispos* **36**:71-92.

Vasange M (2014) Assay suitability for natural product screening: searching for leads to fight Alzheimer's disease. *Planta Med* **80**:1200-1209.

Vasanthanathan P, Hritz J, Taboureau O, Olsen L, Jorgensen FS, Vermeulen NP and Oostenbrink C (2009) Virtual screening and prediction of site of metabolism for cytochrome P450 1A2 ligands. *J Chem Inf Model* **49**:43-52.

Weisburger E (2000) History and background of the threshold limit value (TLV) committee of the ACGIH. *Abstracts of Papers of the American Chemical Society* **220**:U190-U190.

Weisse AB (2010) A fond farewell to the foxglove? The decline in the use of digitalis. *J Card Fail* **16**:45-48.

Wen X, Wang JS, Neuvonen PJ and Backman JT (2002) Isoniazid is a mechanism-based inhibitor of cytochrome P450 1A2, 2A6, 2C19 and 3A4 isoforms in human liver microsomes. *Eur J Clin Pharmacol* **57**:799-804.

Venkatakrishnan K and Obach RS (2007) Drug-drug interactions via mechanism-based cytochrome P450 inactivation: Points to consider for risk assessment from in vitro data and clinical pharmacologic evaluation. *Curr Drug Metab* **8**:449-462.

Werck-Reichhart D and Feyereisen R (2000) Cytochromes P450: a success story. *Genome Biol* **1**:Reviews3003.

Verpoorte R (2000) Pharmacognosy in the new millennium: leadfinding and biotechnology. *J Pharm Pharmacol* **52**:253-262.

Wester MR, Yano JK, Schoch GA, Yang C, Griffin KJ, Stout CD and Johnson EF (2004) The structure of human cytochrome P450 2C9 complexed with flurbiprofen at 2.0-A resolution. *J Biol Chem* **279**:35630-35637.

White RE (2000) High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. *Annu Rev Pharmacol Toxicol* **40**:133-157.

WHO (2004) New alternative medicine guide launched amidst increasing reports of adverse reactions. *Bull World Health Organ* **82**:635-636.

Wible BA, Hawryluk P, Ficker E, Kuryshev YA, Kirsch G and Brown AM (2005) HERG-Lite: a novel comprehensive high-throughput screen for drug-induced hERG risk. *J Pharmacol Toxicol Methods* **52**:136-145.

Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4**:825-833.

Wilkinson CF, Murray M and Marcus CB (1984) Interactions of methylenedioxyphenyl compounds with cytochrome P-450 and effects on microsomal oxidation. *Reviews in Biochemical Toxicology* **6**:27-63.

Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovic D and Jhoti H (2003) Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature* **424**:464-468.

Wimalasena K and Haines DC (1996) A general progress curve method for the kinetic analysis of suicide enzyme inhibitors. *Anal Biochem* **234**:175-182.

Winzer T, Kern M, King AJ, Larson TR, Teodor RI, Donninger SL, Li Y, Dowle AA, Cartwright J, Bates R, Ashford D, Thomas J, Walker C, Bowser TA and Graham IA (2015) Plant science. Morphinan biosynthesis in opium poppy requires a P450-oxidoreductase fusion protein. *Science* **349**:309-312.

Wrighton SA, Stevens JC, Becker GW and Vandenbranden M (1993) ISOLATION AND CHARACTERIZATION OF HUMAN LIVER CYTOCHROME-P450 2C19 - CORRELATION BETWEEN 2C19 AND S-MEPHENYTOIN-4'-HYDROXYLATION. *Arch Biochem Biophys* **306**:240-245.

Wu JJ, Ai CZ, Liu Y, Zhang YY, Jiang M, Fan XR, Lv AP and Yang L (2012) Interactions between phytochemicals from traditional Chinese medicines and human cytochrome P450 enzymes. *Curr Drug Metab* **13**:599-614.

Yamano K, Yamamoto K, Katashima M, Kotaki H, Takedomi S, Matsuo H, Ohtani H, Sawada Y and Iga T (2001) Prediction of midazolam-CYP3A inhibitors interaction in the human liver from in vivo/in vitro absorption, distribution, and metabolism data. *Drug Metab Dispos* **29**:443-452.

Yan Z and Caldwell GW (2001) Metabolism profiling, and cytochrome P450 inhibition & induction in drug discovery. *Curr Top Med Chem* **1**:403-425.

Yan Z and Caldwell GW (2012) The current status of time dependent CYP inhibition assay and in silico drug-drug interaction predictions. *Curr Top Med Chem* **12**:1291-1297.

Yan Z, Rafferty B, Caldwell GW and Masucci JA (2002) Rapidly distinguishing reversible and irreversible CYP450 inhibitors by using fluorometric kinetic analyses. *Eur J Drug Metab Pharmacokinet* **27**:281-287.

Yao M, Cai H and Zhu M (2012) Fast and reliable CYP inhibition assays, in ADME-Enabling Technologies in Drug Design and Development (Zhang D, Surapaneni S ed) pp 213-232, John Wiley & Sons, Inc., New Jersey.

Yates P, Eng H, Di L and Obach RS (2012) Statistical methods for analysis of time-dependent inhibition of cytochrome p450 enzymes. *Drug Metab Dispos* **40**:2289-2296.

Yucel E, Sancar M, Yucel A and Okuyan B (2016) Adverse drug reactions due to drug-drug interactions with proton pump inhibitors: assessment of systematic reviews with AMSTAR method. *Expert Opin Drug Saf* **15**:223-236.

Zanger UM (2012) Cytochrome P450 Polymorphisms, in Encyclopedia of Drug Metabolism and Interactions (Lyubimov A ed) pp 239-274, John Wiley & Sons, New Jersey.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* **138**:103-141.

Zanger UM, Turpeinen M, Klein K and Schwab M (2008) Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem* **392**:1093-1108.

Zenk MH and Juenger M (2007) Evolution and current status of the phytochemistry of nitrogenous compounds. *Phytochemistry* **68**:2757-2772.

Zhang L, Reynolds KS, Zhao P and Huang SM (2010) Drug interactions evaluation: an integrated part of risk assessment of therapeutics. *Toxicol Appl Pharmacol* **243**:134-145.

Zhang N, Seguin RP, Kunze KL, Zhang YY and Jeong H (2013) Characterization of inhibition kinetics of (S)-warfarin hydroxylation by noscapine: implications in warfarin therapy. *Drug Metab Dispos* **41**:2114-2123.

Zhao Y, Hellum BH, Liang A and Nilsen OG (2015) Inhibitory Mechanisms of Human CYPs by Three Alkaloids Isolated from Traditional Chinese Herbs. *Phytother Res* **29**:825-834.

Zhou ZW and Zhou SF (2009) Application of mechanism-based CYP inhibition for predicting drug-drug interactions. *Expert Opin Drug Metab Toxicol* **5**:579-605.

Ziegler J and Facchini PJ (2008) Alkaloid biosynthesis: Metabolism and trafficking. *Annual Review of Plant Biology* **59**:735-769.

Ziegler J, Facchini PJ, Geissler R, Schmidt J, Ammer C, Kramell R, Voigtlander S, Gesell A, Pienkny S and Brandt W (2009) Evolution of morphine biosynthesis in opium poppy. *Phytochemistry* **70**:1696-1707.

Zimmerlin A, Trunzer M and Faller B (2011) CYP3A time-dependent inhibition risk assessment validated with 400 reference drugs. *Drug Metab Dispos* **39**:1039-1046.

Zomorodi K and Houston JB (1995) Effect of omeprazole on diazepam disposition in the rat: in vitro and in vivo studies. *Pharm Res* **12**:1642-1646.

Zou K, Li Z, Zhang Y, Zhang HY, Li B, Zhu WL, Shi JY, Jia Q and Li YM (2017) Advances in the study of berberine and its derivatives: a focus on anti-inflammatory and anti-tumor effects in the digestive system. *Acta Pharmacol Sin* **38**:157-167.

ORIGINAL PUBLICATIONS (I-IV)

I

Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids

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Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids

Kaisa A. Salminen[a,*], Achim Meyer[b], Lenka Jerabkova[a], Laura E. Korhonen[a], Minna Rahnasto[a], Risto O. Juvonen[a], Peter Imming[b], Hannu Raunio[a]

[a] School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland
[b] Institut für Pharmazie, Abteilung Pharmazeutische Chemie, Martin-Luther-Universität Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06120 Halle, Germany

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ABSTRACT

The human cytochrome P450 (CYP) enzymes play a major role in the metabolism of endobiotics and numerous xenobiotics including drugs. Therefore it is the standard procedure to test new drug candidates for interactions with CYP enzymes during the preclinical development phase. The purpose of this study was to determine *in vitro* CYP inhibition potencies of a set of isoquinoline alkaloids to gain insight into interactions of novel chemical structures with CYP enzymes. These alkaloids ($n = 36$) consist of compounds isolated from the *Papaveraceae* family ($n = 20$), synthetic analogs ($n = 15$), and one commercial compound. Their inhibitory activity was determined towards all principal human drug metabolizing CYP enzymes: 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4. All alkaloids were assayed *in vitro* in a 96-well plate format using pro-fluorescent probe substrates and recombinant human CYP enzymes. Many of these alkaloids inhibited the CYP3A4 form, with 30/36 alkaloids inhibiting CYP3A4 with at least moderate potency ($IC_{50} < 10\ \mu M$) and 15/36 inhibiting CYP3A4 potently ($IC_{50} < 1\ \mu M$). Among them corydine, parfumine and 8-methyl-2,3,10,11-tetraethoxyberbine were potent and selective inhibitors for CYP3A4. CYP2D6 was inhibited with at least moderate potency by 26/34 alkaloids. CYP2C19 was inhibited by 15/36 alkaloids at least moderate potently, whereas CYP1A2, CYP2B6, CYP2C8, and CYP2C9 were inhibited to a lesser degree. CYP2A6 was not significantly inhibited by any of the alkaloids. The results provide initial structure–activity information about the interaction of isoquinoline alkaloids with major human xenobiotic-metabolizing CYP enzymes, and illustrate potential novel structures as CYP form-selective inhibitors.

Introduction

Plant alkaloids, one of the largest groups of natural products, represent a highly diverse group of chemical entities. Plants are estimated to produce approximately 12,000 different alkaloids with a wide range of pharmacological properties. Alkaloids can be classified according to their basic heterocyclic ring system. Benzylisoquinoline alkaloids are a group of nitrogen-containing plant secondary metabolites of which approximately 2500 members have been identified. Many of these compounds possess potent pharmacological effects. For example, the well known plant alkaloids include the narcotic analgesics, morphine and codeine, apomorphine (a derivative of morphine) used in Parkinson's disease, the muscle relaxant papaverine, and the antimicrobial agents sanguinarine and berberine. Also several potent anti-cancer drugs have been developed from plant compounds (Stevigny et al. 2005; Ziegler and Facchini 2008; Ziegler et al. 2009). In addition, several Stephania and Corydalis species are used in Traditional Chinese Medicine (TCM) because of their alkaloid content (Mo et al. 2007).

The cytochrome P450 (CYP) enzymes constitute a superfamily of heme-containing mono-oxygenases that catalyse the oxidative metabolism of a wide variety of xenobiotics, including drugs, plant-derived or fungal-derived secondary metabolites consumed with food, and a large number of environmental pollutants, industrial compounds, herbicides, and pesticides. The human CYP forms that metabolize xenobiotics belong to the families CYP1, CYP2 and CYP3. Individual CYP enzymes in these families have broad and overlapping substrate specificities, and are responsible for the metabolism of approximately 70–80% of all currently used drugs (Nebert and Russell 2002; Guengerich et al. 2005; Ingelman-Sundberg 2005). CYP enzymes also play a key role in oxidative reactions in plant secondary metabolism (Ziegler and Facchini 2008), and thus plants have remarkably high numbers of *CYP* genes (Nielsen and Møller 2005).

* Corresponding author. Tel.: +358 40 355 3774; fax: +358 17 162424.
E-mail address: kaisa.salminen@uef.fi (K.A. Salminen).

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Table 1
Experimental conditions in human recombinant CYP enzyme inhibition assays.

CYP	Substrate (μM)	Fluorescent metabolite	Incubation time (min)	Enzyme (pmol)	Excitation/emission (nm)
1A2	7-Ethoxyresorufin (1)	Resorufin	20	0.5	570/615
2A6	Coumarin (10)	7-Hydroxycoumarin	10	0.3	355/460
2B6	EFC (2.5)	HFC	30	0.75	405/535
2C8	DBF (0.5)	Fluorescein	30	1.5	485/535
2C9	MFC (75)	HFC	45	1.5	405/535
2C19	DBF (0.5)	Fluorescein	30	1.5	485/535
2D6	MAMC (20)	HAMC	60	2.0	405/460
3A4	MFC (50)	HFC	30	1.0	405/535

Abbreviations: EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; DBF, dibenzylfluorescein; MFC, 7-methoxy-4-(trifluoromethyl)coumarin; BFC, 7-benzyloxy-4-(trifluoromethyl)coumarin; MAMC, 7-methoxy-4-(aminomethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; HAMC, 7-hydroxy-4-(aminomethyl)coumarin.

To a large extent metabolism determines the pharmacokinetic behaviour of a drug, i.e., the intensity and the duration of action. Modulation of CYP activity via inhibition or induction by drugs and other xenobiotics often is the source of drug interactions. Drug interactions can evoke severe adverse effects, they have resulted in early termination of drug development, refusal to obtain approval, severe prescribing restrictions, and even withdrawal of drugs from the market. The most common mechanism of drug interactions is inhibition of these CYP enzymes (Kalgutkar et al. 2007; Pelkonen et al. 2008). On the other hand, specific CYP inhibitors that do not interact with other targets and have a clear pharmacodynamic profile are potential co-therapeutics, especially in the field of antivirals. The so-called pharmacokinetic boosters inhibit the CYPs metabolizing antiviral drugs, e.g. the HIV-1 protease inhibitor lopinavir, is used to raise the effective concentration of the antiviral drug in the body. The advantages of this approach range from possible reductions in drug load to improvements of patient compliance by achieving longer dosing intervals (Dickinson et al. 2010).

In early drug development, experiments are routinely carried out to determine which CYP enzymes catalyse the metabolism of lead compounds. In addition, the potential of lead compounds to inhibit CYPs can be evaluated with *in vitro* methods. Often, but not always, a compound that inhibits a specific CYP form is also a substrate for that same form. These experiments employ various sources of CYP enzymes, e.g. human liver or cDNA-expressed human enzymes, and probe substrates and inhibitors (Bjornsson et al. 2003; van de Waterbeemd and Gifford 2003; Pelkonen and Raunio 2005).

There is still plenty of scope to enlarge the known chemical space of CYP inhibitors and substrates. The purpose of this study was (1) to screen a series of novel plant-derived isoquinoline alkaloids for their abilities to interact with the most important human liver xenobiotic-metabolizing CYP enzymes, and (2) to search for novel CYP form-selective inhibitory compounds. The inhibition potency of eight protopine, six aporphine, one spirobenzylisoquinoline and one phthalideisoquinoline, and 20 protoberberine alkaloids was determined against recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes.

Materials and methods

Chemicals and reagents

The origin of the tested alkaloids is the following (numbering according to Table 2). Alkaloid **1** was isolated by W. H. Perkin in 1918 from *Bocconia cordata* Willd. Alkaloid **2** was isolated by J. Slavik in 1966 from *Hunnemannia fumariifolia* Sweet. Alkaloid **3** was isolated by W. H. Perkin in 1926 from *Papaver somniferum* L. Alkaloid **4** was isolated by J. Slavik from *Chelidonium majus* L. Alkaloid **27** was isolated by J. Slavik from *Meconopsis cambrica* (L.) Vig. Alkaloids **5**, **6**, **8**–**14** and **19**–**26** were from a historical collection gathered by Johannes Gadamer and co-workers at the beginning of the 20th century. Alkaloid **8** thereof is the semisynthetic derivative of **9**. Alkaloids **5**, **6**, **9**–**12**, **14** and **19**–**26** were isolated from *Corydalis cava* L. and alkaloid **13** from *Fumaria vaillantii Loisel*. Alkaloids **15**–**18** are semisynthetic and alkaloids **28**–**36** synthetic (Meyer et al. unpublished). Alkaloid **7** was purchased from Sigma Aldrich (St. Louis, MO, USA). The purity of the alkaloids was higher than 95% as determined by gas chromatography–mass spectrometry and combustion analysis. All structures were confirmed by ^{1}H NMR and mass spectrometry.

cDNA-expressed human wild-type CYPs (Supersomes^{TM}) were purchased from BD Biosciences Discovery Labware (Bedford, MA, USA). Substrates and metabolite standards were purchased from BD Biosciences Discovery Labware (Bedford, MA) and Sigma Chemical Company (St. Louis, MO). These were 7-ethoxyresorufin and resorufin for CYP1A2; coumarin and 7-hydroxycoumarin for CYP2A6; 7-ethoxy-4-(trifluoromethyl)coumarin (EFC) and 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) for CYP2B6; dibenzylfluorescein (DBF) and fluorescein for CYP2C8 and CYP2C19; 7-methoxy-4-(trifluoromethyl)coumarin (MFC) and HFC for CYP2C9 and CYP3A4; and 7-methoxy-4-(aminomethyl)coumarin (MAMC) and 7-hydroxy-4-(aminomethyl)coumarin (HAMC) for CYP2D6. All other chemicals used were from Sigma–Aldrich (St. Louis, MO, USA) and were of the highest purity available.

CYP inhibition assays

Incubations were conducted in a 150 μl volume in 96-well microtiter plates based on the general principles originally published by Crespi et al. (1997), using cDNA-expressed recombinant CYP enzymes. Each test compound was screened using four concentrations (mainly 1:10 ratio) ranging from 0.01 to 1000 μM in a duplicate layout. The incubation mixtures contained 100 mM Tris/HCl buffer (pH 7.4), 0.75–2.0 pmol of CYP enzyme, the probe substrate at the concentration corresponding to its measured apparent K_m, and the nicotine adenine dinucleotide phosphate hydrogen (NADPH)-regenerating system (consisting of 1.13 mM NADP, 12.5 mM isocitric acid, 56.33 mM KCl, 187.5 mM Tris/HCl, pH 7.4, 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$, 0.075 U ml^{-1} isocitrate dehydrogenase), except 50 mM Tris/HCl buffer (pH 7.4), 0.3 mM NADPH, and 5 mM $MgCl_2$ in a 100 μl incubation volume in the case of CYP2A6. Due to their high lipophilicity the test compounds were dissolved in acetonitrile (ACN) or dimethyl sulfoxide (DMSO) and then further diluted with water. Consequently, the final solvent concentrations in the incubations did not exceed 2%. Only the hydrochloride salts were directly soluble in water. Controls were treated similarly but without the presence of inhibitors. The reactions were initiated by adding 50 μl of the NADPH-regenerating system, except for 25 μl of 1.2 mM NADPH in the case of CYP2A6, after a 10-min preincubation at 37 °C. After incubation (10–60 min), the reactions were terminated by addition of the stop solution (Table 1). In addition, in the case of CYP2A6, immediately before the measurement, 140 μl of 1.6 M glycine–NaOH buffer (pH 10.4) was

Table 2
CYP inhibition screening results organized according to the isoquinoline alkaloid subgroups.

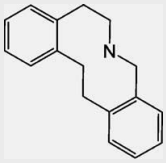
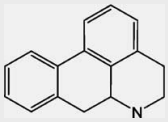
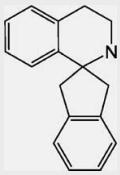
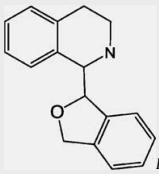
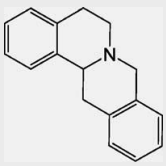
No.	Compound	1A2	2A6	2B6	2C8	2C9	2C19	2D6	3A4
	 *Protopine alkaloids*								
1	Protopine	++	–	+	–	–	++	+++	+
2	Hunnemannine	+	–	+	++	–	+	+++	++
3	Cryptopine	–	–	–	+	–	++	+++	–
4	Allocryptopine	+	–	+	+	–	–	+++	++
5	Corycavidine	–	–	+	+	–	+	++	++
6	Corycavamine	+	–	+	+	–	++	+++	++
15	Dihydrocryptopine	+	–	–	+	–	++	++	+++
16	Dihydroprotopine	+	–	++	+	++	+++	+++	+++
	 *Aporphine alkaloids*								
7	Apomorphine HCl	++	–	+	++	++	+	+	+
8	Bulbocapninemethylether	++	–	+	–	+	+	++	+++
9	Bulbocapnine	++	–	+	–	+	+	++	+++
10	Isocorydine	+	–	+	–	+	+	+	++
11	Corydine	+	–	+	–	+	+	+	+++
12	Nantenine	+++	–	+	++	++	++	ND	+++
	 *Spirobenzylisoquinoline alkaloide*								
13	Parfumine	+	–	+	–	+	+	+	+++
	 *Phthalideisoquinoline alkaloide*								
14	Capnoidine	+	–	–	+	–	+++	+++	+++
	 *Protoberberine alkaloids*								
17	Canadine	++	–	++	–	–	++	+++	+++
18	Nandinine	++	–	++	+	+	++	+++	++
19	Corydaline	+	–	–	+	++	++	+	+++
20	Tetrahydropalmatine	+	–	–	–	+	+	+++	++
21	Corypalmine	+	–	–	+	+	++	++	+++
22	Stylopine	++	–	+	–	++	+++	+++	+++
23	Thalictricavine	++	–	++	+	–	+	ND	+
24	Isocorybulbine	+	–	–	+	+	–	+	++
25	Corybulbine	+	+	–	+	+	+	–	++
26	Scoulerine HCl	+	–	+	+	+	+	+++	++
27	Mecambridine	+	–	–	+	+	–	+++	++
28	2,3,10,11-Tetramethoxyberbine	+	–	–	–	–	–	+++	++
29	8-Ethyl-2,3,10,11-tetramethoxyberbine	+	–	+	–	–	–	+++	++
30	8-Methyl-2,3,10,11-tetramethoxyberbine	+	–	–	–	++	+	+++	++

Table 2 *(Continued.)*

No.	Compound	1A2	2A6	2B6	2C8	2C9	2C19	2D6	3A4
31	8-Methyl-2,3,10,11-tetraethoxyberbine	+	–	+	+	–	+	+	+++
32	2,3-Dimethoxy-10,11-methylenedioxyberbine	+	–	–	–	–	++	+++	++
33	2,3-Dimethoxy-9,10-methylenedioxyberbine	+	–	+	+	+	+	+++	+++
34	3,10,11-Trimethoxyberbine	+	–	+	–	–	++	+++	+++
35	3-Methoxyberbine	++	–	+	–	–	++	+++	–
36	2,3-Dimethoxyberbine HCl	++	–	–	–	+	+	+++	+

+++, IC_{50} < 1 μM; ++, IC_{50} 1–10 μM; +, IC_{50} 10–100 μM; –, IC_{50} > 100 μM; *ND* not determined.

added into the wells. The experimental conditions are summarized in Table 1.

Fluorescence was measured with a Victor^2 plate counter (Perkin-Elmer Life Sciences Wallac, Turku, Finland) with excitation and emission wavelengths set according to the probe substrate/metabolite. The IC_{50} values (inhibitor concentration that reduced the metabolism of the CYP probe substrate by 50%) were calculated using non-linear regression analysis with Prism 4.0 software (San Diego, CA, USA).

The linearity of the reactions with respect to incubation time and protein concentration as well as the basic kinetic parameters (K_m and V_{max}) for the probe substrates was determined before the actual experiments (data not shown). In addition, several control incubations (with positive controls) were carried out to confirm the reliability of the assays, as described previously (Turpeinen et al. 2006). The extent of inhibition was classified as potent (IC_{50} < 1 μM), marginal/moderate (1 μM < IC_{50} < 10 μM), weak (IC_{50} > 10 μM), or no inhibition (IC_{50} > 100 μM) (White 2000).

Results

The capabilities of plant isoquinoline alkaloids to inhibit individual CYP enzymes, i.e., changes caused by the alkaloids in the metabolism of CYP-specific probe substrates, were determined with eight recombinant human CYPs. The alkaloids were classified to four groups according to their inhibition potency: potent IC_{50} < 1 μM, marginal/moderate 1 μM < IC_{50} < 10 μM, weak IC_{50} > 10 μM, or no inhibition IC_{50} > 100 μM (White 2000). The screening results for all alkaloids and CYP forms are presented in Table 2. A summary of the number and the percentage of alkaloids in each inhibition potency category are provided in Table 3. The results revealed that the majority of alkaloids inhibited most potently the CYP3A4 form (30/36 with at least moderate potency and 15/36 potently), and CYP2D6 (26/34 with at least moderate potency and 21/34 potently), and to some extent also CYP2C19 (15/36 with at least moderate potency and 3/36 potently). The alkaloids had a clearly less inhibitory effect on CYP2C8 (3/36 with moderate potency), CYP2B6 (4/36 with moderate potency), and CYP2C9 (6/36 with moderate potency). Furthermore, 10/36 alkaloids inhibited CYP1A2 with moderate potency and 1/36 (nantenine) potently. The tested alkaloids did not inhibit CYP2A6.

CYP2D6 was inhibited most potently by the protopine alkaloids (6/8 potently) and protoberberine alkaloids (14/20 potently) as well as the phthalideisoquinoline alkaloide capnoidine. The aporphine alkaloids and the spirobenzylisoquinoline alkaloide, parfumine, inhibited CYP2D6 only slightly (2/6 aporphine alkaloids with moderate potency). In contrast to CYP2D6, CYP3A4 was inhibited by the aporphine alkaloids. Protopine and protoberberine alkaloids were more potent inhibitors of CYP2D6 than CYP3A4. Alkaloids inhibiting CYP2C19 belonged mostly to the protopine and protoberberine alkaloids.

Fig. 1 illustrates the inhibition of CYP1A2, CYP2A6, CYP2D6, and CYP3A4 by apomorphine (**7**). Apomorphine was a moderately potent inhibitor of CYP1A2 with an IC_{50} value of 4.8 ± 1.8 μM (mean ± SEM), and a weak inhibitor of CYP2D6 and CYP3A4 with IC_{50} values of 11.3 ± 5.5 μM and 34.0 ± 8.1 μM, respectively. In contrast, apomorphine was not able to inhibit CYP2A6 (IC_{50} > 100 μM).

Discussion

The results of this study provide an extensive perspective of the interaction of novel plant isoquinoline alkaloids with the most important human CYP enzymes. Overall, there were clear differences in the ability of these alkaloids to inhibit individual CYP forms. CYP2D6 and CYP3A4 were most potently inhibited, whereas CYP1A2, CYP2B6, CYP2C8, CYP2C9, and CYP2C19 were inhibited to a lesser degree. One notable feature was the inability of the alkaloids to affect CYP2A6. This is consistent with the known small and restricted active site of this CYP form (Yano et al. 2006). Several new potent inhibitors against CYP3A4, CYP2D6, CYP2C19, and CYP1A2 were found. Of particular interest is the selective inhibition of CYP3A4 by corydine (**11**), parfumine (**13**) and 8-methyl-2,3,10,11-tetraethoxyberbine (**31**).

Fluorometric high-throughput inhibition assays of CYP enzymes are widely used for drug interaction screening particularly during the preclinical drug discovery stages. Fluorometric inhibition screening has been found to be comparable with other potential screening procedures employed, such as HPLC and mass spectrometry detection (Turpeinen et al. 2006; Pelkonen et al. 2008; Kapitulnik et al. 2009). When many compounds of unknown and varying inhibitory potency are being screened, the IC_{50} value is a practical readout of the relative effects on CYP enzyme activity under well-controlled conditions. By using suitable *in vitro* probes and careful selection of interacting drugs during *in vivo* studies, the potential for drug interactions can be evaluated early in the development process (Huang et al. 2007). Therefore a fluorometric high-throughput inhibition assay was used in this study to determine interaction potency between CYPs and plant isoquinoline alkaloids.

CYP3A4 has a pivotal role in xenobiotic metabolism, and it has been estimated to be involved in the metabolism of up to 50% of all drugs in clinical use. The active site of CYP3A4 is very large and flexible allowing the binding and subsequent metabolism of structurally very diverse compounds. The substrate binding is principally based on hydrophobicity with some steric interactions (Williams et al. 2004; Yano et al. 2004; Zhou 2008). In this study, 42% of the tested isoquinoline alkaloids were potent and 42% moderate inhibitors of CYP3A4. The current findings will add to current understanding of the structure–activity relationship of ligands at the active site of CYP3A4. Corydine (**11**), parfumine (**13**), and 8-methyl-2,3,10,11-tetraethoxyberbine (**31**) are novel CYP3A4-selective inhibitory compounds. They inhibited potently CYP3A4 and weakly or not at all the other CYP forms. Therefore they may be used as selective inhibitors of CYP3A4 to study CYP mediated metabolism of test compounds *in vitro*.

The typical CYP2D6 substrates are lipophilic bases with a planar hydrophobic aromatic ring and a nitrogen atom which can be protonated at physiological pH (Lewis 2004). The tested isoquinoline alkaloids possess these properties. Lipophilicity and amine basicity are considered to be the two critical determinants of

Table 3
Summary of the isoquinoline alkaloids in each inhibition potency category.

Category (IC_{50} value)	CYP2D6 $n = 34$ (%)	CYP3A4 $n = 36$ (%)	CYP2C19 $n = 36$ (%)	CYP1A2 $n = 36$ (%)	CYP2C9 $n = 36$ (%)	CYP2B6 $n = 36$ (%)	CYP2C8 $n = 36$ (%)	CYP2A6 $n = 36$ (%)
Potent (<1 μM)	21 (61.8)	15 (41.7)	3 (8.3)	1 (2.8)	0 (0)	0 (0)	0 (0)	0 (0)
Moderate (1–10 μM)	5 (14.7)	15 (41.7)	12 (33.3)	10 (27.8)	6 (16.7)	4 (11.1)	3 (8.3)	0 (0)
Weak (>10 μM)	7 (20.6)	4 (11.1)	16 (44.4)	23 (63.9)	14 (38.9)	19 (52.8)	17 (47.2)	1 (2.8)
Non-inhibitor (>100 μM)	1 (2.9)	2 (5.5)	5 (13.9)	2 (5.5)	16 (44.4)	13 (36.1)	16 (44.4)	35 (97.2)

The CYP forms most potently inhibited are presented first.

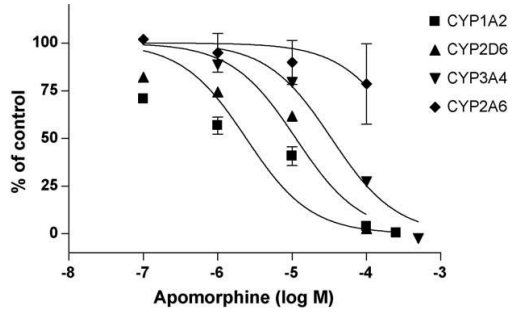


Fig. 1. Inhibition of CYP1A2, CYP2D6, CYP3A4, and CYP2A6 by apomorphine. Percentages of remaining activity for each CYP form are plotted as a function of apomorphine concentration. Non-linear curve fitting was used to create the inhibition curves.

substrate binding to CYP2D6. A number of CYP2D6 substrates and other compounds have been found to inhibit CYP2D6 (Lewis 2004; Wang et al. 2009). In this study, as high as 62% of the alkaloids were potent inhibitors of CYP2D6. However, none of them was as selective as the three found inhibitors of CYP3A4. However, cryptopine (**3**), allocryptopine (**4**), scoulerine (**26**), mecambridine (**27**), 2,3,10,11-tetramethoxyberbine (**28**), 8-ethyl-2,3,10,11-tetramethoxyberbine (**29**) and 2,3-dimethoxyberbine (**36**) had some selectivity towards CYP2D6, and these compounds add to the list of known CYP2D6 ligands (Lewis 2004; Wang et al. 2009).

The diversity of the biosynthetic pathways in plants has provided a variety of lead structures that have been used in drug development and which have been estimated to account for more than 50% of current drugs. The plant kingdom still remains a treasure trove of new molecules with therapeutic potential (Stevigny et al. 2005; Newman and Cragg 2007). Alkaloids exist in a number of herbal medicines as their major biologically active constituents. Herbal alkaloids may be substrates, inducers, or inhibitors of various CYPs. For example, some vinca alkaloids (e.g. vinblastine) are metabolized by CYP3A4, and this has been associated with tumour resistance (Yao et al. 2000; Zhou et al. 2003).

Apomorphine was the only compound in this study currently used as a drug. It is administered subcutaneously to treat Parkinson's disease. Despite being a derivative of the opioid, morphine, apomorphine lacks the narcotic properties and other opiate effects of its parent compound. Apomorphine is not commercially available for oral administration because of its extensive inactivation via hepatic first-pass metabolism and poor bioavailability. The route of metabolism after subcutaneous administration of apomorphine in humans is not known but in several animal species, apomorphine undergoes metabolism by glucuronidation and *O*-methylation (Chen and Obering 2005). In this study, apomorphine did not possess any significant inhibitory capabilities against human CYPs (moderate potency towards CYP1A2, CYP2C8, and CYP2C9, and weak potency towards CYP2B6, CYP2C19, CYP2D6 and CYP3A4). For CYP1A2, CYP2D6, and CYP3A4, these results are consistent with those obtained with human hepatic microsomes *in vitro* (Argiolas and Hedlund 2001).

The prodrug codeine is extensively metabolized by CYP2D6, and the clinical analgesic effect of codeine is mainly attributed to its conversion to morphine (Lotsch et al. 2009). Like apomorphine, also buprenorphine (a semisynthetic opioid derived from thebaine, naturally occurring alkaloid of Papaver somniferum, and used as substitution therapy in the treatment of opioid dependence), undergoes extensive first-pass metabolism and therefore has very low oral bioavailability (Elkader and Sproule 2005). In

addition, it is known that buprenorphine is extensively metabolized by *N*-dealkylation primarily via CYP3A4 (Elkader and Sproule 2005).

To learn whether the current alkaloids are present in TCM, a search of a commercial TCM Database (NiceData Software 2005) was carried out. This database includes 10,458 individual compounds isolated from TCM plants, 4636 TCM medicinal plants, and their therapeutic utilities. Of the present 36 alkaloids, 19 were found in the database. For 10 alkaloids (**1**, **3**, **4**, **9**, **10**, **11**, **14**, **17**, **27**, and **28**) one or more TCM pharmacological or therapeutic effects were listed. Three plants listed in the database (*Papaver somniferum* L., *Chelidonium majus* L., and *Corydalis cava* L.) were related to alkaloids **1**, **3**, **4**, **9**, **10**, **17**, and **22**. In contrast to TCM, isoquinoline-containing plants are currently not commonly used in European medicine, as exemplified by the fact that the European Pharmacopoeia (Ph. Eur.) includes only *Fumaria officinalis* L.

In conclusion, this study presents a detailed account on the ability of a series of structurally related alkaloids to interact with all major human metabolizing CYP enzymes. To date, here is only a sparse literature on interactions between plant alkaloids and human CYP enzymes. However, there is information about the preferred substrates and the functionality of the major human CYP enzymes and these present results agree with these general principles. The current study indicated high-affinity interaction of several isoquinoline alkaloids to several CYP enzymes. Future experiments on the actual metabolic turnover of the compounds will yield insight into their possible metabolic fate in humans. This dataset also provides an opportunity to carry out detailed structure–activity relationship studies. Several novel compounds with selectivities towards individual CYP forms were identified and these have the potential to be developed as form-specific inhibitors for further *in vitro* CYP screenings.

Conflicts of interest

The authors have no conflicts to disclose.

Acknowledgements

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References

Argiolas, A., Hedlund, H., 2001. The pharmacology and clinical pharmacokinetics of apomorphine SL. BJU Int. 88, 18–21.

Bjornsson, T.D., Callaghan, J.T., Einolf, H.J., Fischer, V., Gan, L., Grimm, S., Kao, J., King, S.P., Miwa, G., Ni, L., Kumar, G., McLeod, J., Obach, S.R., Roberts, S., Roe, A., Shah, A., Snikeris, F., Sullivan, J.T., Tweedie, D., Vega, J.M., Walsh, J., Wrighton, S.A., 2003. The conduct of in vitro and in vivo drug–drug interaction studies: a PhRMA perspective. J. Clin. Pharmacol. 43, 443–469.

Chen, J.J., Obering, C., 2005. A review of intermittent subcutaneous apomorphine injections for the rescue management of motor fluctuations associated with advanced Parkinson's disease. Clin. Ther. 27, 1710–1724.

Crespi, C.L., Miller, V.P., Penman, B.W., 1997. Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. Anal. Biochem. 248, 188–190.

Dickinson, L., Khoo, S., Back, D., 2010. Pharmacokinetics and drug–drug interactions of antiretrovirals: an update. Antiviral Res. 85, 176–189.

Elkader, A., Sproule, B., 2005. Bluprenorphine – clinical pharmacokinetics in the treatment of opioid dependence. Clin. Pharmacokinet. 44, 661–680.

Guengerich, F.P., Wu, Z.L., Bartleson, C.J., 2005. Function of human cytochrome P450s: characterization of the orphans. Biochem. Biophys. Res. Commun. 338, 465–469.

Huang, S.M., Temple, R., Throckmorton, D.C., Lesko, L.J., 2007. Drug interaction studies: study design, data analysis, and implications for dosing and labeling. Clin. Pharmacol. Ther. 81, 298–304.

Ingelman-Sundberg, M., 2005. The human genome project and novel aspects of cytochrome P450 research. Toxicol. Appl. Pharmacol. 207, 52–56.

Kalgutkar, A.S., Obach, R.S., Maurer, T.S., 2007. Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure–activity relationships and relationship to clinical drug–drug interactions and idiosyncratic adverse drug reactions. Curr. Drug Metab. 8, 407–447.

Kapitulnik, J., Pelkonen, O., Gundert-Remy, U., Dahl, S.G., Boobis, A.R., 2009. Effects of pharmaceuticals and other active chemicals at biological targets: mechanisms, interactions, and integration into PB-PK/PD models. Expert Opin. Ther. Targets 13, 867–887.

Lewis, D.F.V., 2004. 57 varieties: the human cytochromes P450. Pharmacogenomics 5, 305–318.

Lotsch, J., Geisslinger, G., Tegeder, I., 2009. Genetic modulation of the pharmacological treatment of pain. Pharmacol. Ther. 124, 168–184.

Mo, J., Guo, Y., Yang, Y.S., Shen, J.S., Jin, G.Z., Zhen, X.C., 2007. Recent developments in studies of l-stepholidine and its analogs: chemistry, pharmacology and clinical implications. Curr. Med. Chem. 14, 2996–3002.

Nebert, D.W., Russell, D.W., 2002. Clinical importance of the cytochromes P450. Lancet 360, 1155–1162.

Newman, D.J., Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod. 70, 461–477.

NiceData Software, 2005. Traditional Chinese Medicines Database. Version 2.1. CambridgeSoft Corporation, Cambridge, MA.

Nielsen, K.A., Møller, B.L., 2005. Cytochrome P450s in plants. In: Ortiz de Montellano, P.R. (Ed.), Cytochrome P450: Structures, Mechanism, and Biochemistry. Kluwer Academic/Plenum Publishers, New York, pp. 553–583.

Pelkonen, O., Raunio, H., 2005. In vitro screening of drug metabolism during drug development: can we trust the predictions? Expert Opin. Drug Metab. Toxicol. 1, 49–59.

Pelkonen, O., Turpeinen, M., Hakkola, J., Honkakoski, P., Hukkanen, J., Raunio, H., 2008. Inhibition and induction of human cytochrome P450 enzymes: current status. Arch. Toxicol. 82, 667–715.

Stevigny, C., Bailly, C., Quetin-Leclercq, J., 2005. Cytotoxic and antitumor potentialities of aporphinoid alkaloids. Curr. Med. Chem. Anticancer Agents 5, 173–182.

Turpeinen, M., Korhonen, L.E., Tolonen, A., Uusitalo, J., Juuonen, R., Raunio, H., Pelkonen, A., 2006. Cytochrome P450 (CYP) inhibition screening: comparison of three tests. Eur. J. Pharm. Sci. 29, 130–138.

van de Waterbeemd, H., Gifford, E., 2003. ADMET in silico modelling: towards prediction paradise? Nat. Rev. Drug Discov. 2, 192–204.

Wang, B., Yang, L.P., Zhang, X.Z., Huang, S.Q., Bartlam, M., Zhou, S.F., 2009. New insights into the structural characteristics and functional relevance of the human cytochrome P450 2D6 enzyme. Drug Metab. Rev. 41, 573–643.

White, R.E., 2000. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. Annu. Rev. Pharmacol. Toxicol. 40, 133–157.

Williams, P.A., Cosme, J., Vinkovic, D.M., Ward, A., Angove, H.C., Day, P.J., Vonrhein, C., Tickle, I.J., Jhoti, H., 2004. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. Science 305, 683–686.

Yano, J.K., Denton, T.T., Cerny, M.A., Zhang, X.D., Johnson, E.F., Cashman, J.R., 2006. Synthetic inhibitors of cytochrome P-450 2A6: inhibitory activity, difference spectra, mechanism of inhibition, and protein cocrystallization. J. Med. Chem. 49, 6987–7001.

Yano, J.K., Wester, M.R., Schoch, G.A., Griffin, K.J., Stout, C.D., Johnson, E.F., 2004. The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-angstrom resolution. J. Biol. Chem. 279, 38091–38094.

Yao, D.G., Ding, S.H., Burchell, B., Wolf, C.R., Friedberg, T., 2000. Detoxication of vinca alkaloids by human P450CYP3A4-mediated metabolism: implications for the development of drug resistance. J. Pharmacol. Exp. Ther. 294, 387–395.

Zhou, S.F., 2008. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. Curr. Drug Metab. 9, 310–322.

Zhou, S.F., Gao, Y.H., Jiang, W.Q., Huang, M., Xu, A.L., Paxton, J.W., 2003. Interactions of herbs with cytochrome P450. Drug Metab. Rev. 35, 35–98.

Ziegler, J., Facchini, P.J., 2008. Alkaloid biosynthesis: metabolism and trafficking. Annu. Rev. Plant Biol. 59, 735–769.

Ziegler, J., Facchini, P.J., Geissler, R., Schmidt, J., Ammer, C., Kramell, R., Voigtlander, S., Gesell, A., Pienkny, S., Brandt, W., 2009. Evolution of morphine biosynthesis in opium poppy. Phytochemistry 70, 1696–1707.

II

Simple, direct, and informative method for the assessment of CYP2C19 enzyme inactivation kinetics

Salminen KA, Leppänen J, Venäläinen JI, Pasanen M, Auriola S, Juvonen RO and Raunio H

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Simple, Direct, and Informative Method for the Assessment of CYP2C19 Enzyme Inactivation Kinetics

Kaisa A. Salminen, Jukka Leppänen, Jarkko I. Venäläinen, Markku Pasanen, Seppo Auriola, Risto O. Juvonen, and Hannu Raunio

School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland (K.A.S., J.L., M.P., S.A., R.O.J., H.R.); and Orion Corporation, Orion Pharma, Research and Development, Turku, Finland (J.I.V.)

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ABSTRACT:

Many clinically relevant drug interactions involving cytochrome P450 inhibition are mediated by mechanism-based inactivation (MBI). Time-dependent inhibition is one of the major features distinguishing between reversible inhibition and MBI. It thus provides a useful screening approach for early drug interaction risk assessment. Accordingly, we developed an easy and informative fluorometric method for the assessment of CYP2C19 enzyme inactivation kinetics. Dibenzylfluorescein (DBF) is widely used as a profluorescent probe substrate for P450 activity and inhibition assays, but its use has been considered to be limited to traditional endpoint assays. We monitored CYP2C19-catalyzed metabolism of DBF using synthesized fluorescein benzyl ester and fluorescein benzyl ether along with commercially available fluorescein as intermediate standards. Furthermore, we demonstrated the use of DBF in a kinetic assay as a progress curve analysis for straightforward determination of whether a compound is a time-dependent inactivator of CYP2C19. The recombinant human CYP2C19 inactivation kinetics of isoniazid, ticlopidine, and tranylcypromine were evaluated, and their key kinetic parameters were measured from the same experiment. The known mechanism-based inactivators, isoniazid and ticlopidine, exhibited clear time-dependent inactivation with K_I and k_{inact} values of 250.5 ± 34 μM and 0.137 ± 0.006 min^{-1} and 1.96 ± 0.5 μM and 0.135 ± 0.009 min^{-1}, respectively. Tranylcypromine did not display any time-dependent inhibition, which is consistent with its reported mechanism of competitive inhibition. In summary, DBF is suitable for use in the progress curve analysis approach and can be used as an initial screen to identify compounds that require more detailed investigations in drug interaction optimization.

Introduction

The human cytochrome P450 (P450) enzymes play an important role in the metabolism of drugs and numerous other xenobiotics. Inhibition of P450 enzymes is a common mechanism that can lead to drug interactions. These can evoke severe adverse effects; they have resulted in early termination of drug development, refusal to obtain approval, prescribing restrictions, and even withdrawal of drugs from the market (Wienkers and Heath, 2005; Kalgutkar et al., 2007; Pelkonen et al., 2008).

P450 inhibition can be categorized as either reversible or irreversible. Irreversible inactivation is generally of greater concern than reversible inhibition because it can result in more profound and prolonged effects (Ghanbari et al., 2006; Kalgutkar et al., 2007). There is increasing awareness that many clinically relevant drug interactions involving P450 inhibition are mediated by irreversible mechanism-based inactivation (MBI) (Ghanbari et al., 2006; Grime et al., 2009). Today, potential in vivo effects of drug interactions caused by competitive inhibitors can be fairly well predicted from in vitro P450 kinetics. The current challenge is to detect time- and concentration-dependent effects of irreversible and quasi-irreversible inactivators among large numbers of early-phase compounds in the drug development pipeline (Wienkers and Heath, 2005; Fowler and Zhang, 2008). This information is especially important because failure to consider MBI in vitro can lead to serious underestimation of drug interaction magnitude in vivo, particularly when one is trying to predict drug interactions from in vitro data based on competitive models (Bjornsson et al., 2003; Polasek and Miners, 2007).

The updated regulatory guidances by the U.S. Food and Drug Administration (Guidance for industry: drug interaction studies—study design, data analysis, and implications for dosing and labeling, 2006, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) and European Medicines Agency (Guideline on the investigation of drug interactions, 2010, http://www.emea.europa.eu/htms/human/humanguidelines/efficacy.htm) for in vitro drug interaction studies include recommendations that drug candidates need to be tested for time-dependent and mechanism-based inactivator properties. Recently, a team of scientists from 16 pharmaceutical research organizations recommended the use of a tiered approach wherein abbreviated assays are first used to determine whether or not new chemical entities demonstrate time-dependent inhibition, followed by more thorough inactivation studies for those that do (Grimm et al., 2009).

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ABBREVIATIONS: P450, cytochrome P450; MBI, mechanism-based inactivation; DBF, dibenzylfluorescein; EtOAc, ethyl acetate; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

Current in vitro inactivation research methodologies have been criticized for being significantly influenced by the wide range in experimental conditions, complicating comparison across studies and jeopardizing clinical predictions. In addition, these complex experiments offer limited mechanistic insight (Ghanbari et al., 2006; Riley et al., 2007; Fowler and Zhang, 2008; Obach, 2009; Zhou and Zhou, 2009). Fairman et al. (2007) proposed an alternative in vitro approach for the investigation of pre-steady-state kinetics of CYP1A2 inactivation, referred to as progress curve analysis. Progress curve analysis uses an "all-in" approach in which the enzyme is exposed simultaneously to probe substrate and inactivator while enzyme activity is monitored throughout the inactivation. This type of analysis has long been an accepted tool for measuring pre-steady-state inhibition kinetics for a variety of physiological enzymes (Fairman et al., 2007; Obach, 2009; Zhou and Zhou, 2009).

Dibenzylfluorescein (DBF) is widely used as a profluorescent probe substrate, in particular for CYP2C8, CYP2C9, CYP2C19, CYP3A4, and aromatase (CYP19), in high-throughput assays. CYP2C19 was chosen as the target enzyme in this study because it metabolizes several widely used drugs, such as proton pump inhibitors (Pelkonen et al., 2008). Recent data indicate that inhibition of CYP2C19 may lead to a reduction in clinical efficacy of the antithrombotic prodrug clopidogrel (Wallentin, 2009). The DBF assay is based on the general principles originally published by Crespi and coworkers (Crespi et al., 1997; Crespi and Stresser, 2000). The utility of DBF for kinetic assays with continuous data acquisition has been questioned because the initial metabolite of DBF (fluorescein benzyl ester) requires very alkaline conditions for further hydrolysis to maximize the fluorescence intensity (Crespi and Stresser, 2000; Miller et al., 2001).

The purpose of this study was to 1) characterize the properties of DBF as a probe substrate for CYP2C19 enzyme activity and inhibition assays, 2) assess whether DBF can be used as probe substrate in a real-time kinetic assay, and 3) demonstrate the use of the progress curve analysis approach for rapid identification of time-dependent P450 inactivators as well as the analysis of key inactivation kinetic parameters.

Materials and Methods

Materials. Isoniazid, *trans*-2-phenylcyclopropylamine hydrochloride (tranylcypromine), ticlopidine, and fluorescein were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Dibenzylfluorescein (purity >99%) and cDNA-expressed human wild-type CYP2C19 (Supersomes) were purchased from BD Biosciences Discovery Labware (Bedford, MA). Fluorescein benzyl ester and fluorescein benzyl ether were synthesized with reagents of commercial high purity quality without further purification unless otherwise mentioned. Reactions were monitored by thin-layer chromatography using aluminum sheets coated with Silica Gel 60 F245 (0.24 mm) with suitable visualization. The microwave irradiation experiment was performed in a Biotage Initiator Microwave Reactor (Biotage, Uppsala, Sweden) in a pressure-rated glass tube. Purifications by flash chromatography were performed on Silica Gel 60 (0.063–0.200 mm mesh). ^{1}H and ^{13}C NMR spectra were recorded on an Bruker Avance AV 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at 500.13 and 125.75 MHz, respectively, using tetramethylsilane as an internal standard. The products were also characterized by mass spectrometry with a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with an electrospray ionization source. The purity was determined by elemental analysis (carbon, hydrogen, and nitrogen) with a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer (CE Instruments, Milan, Italy).

Synthesis of Fluorescein Benzyl Ester. Fluorescein (800 mg, 2.4 mmol), benzyl alcohol (10 ml), and concentrated sulfuric acid (1.2 g, 12 mmol) were irradiated with microwaves at 120°C for 1 h (Fig. 1). The reaction mixture was poured into a water-$NaHCO_3$ (50 ml:3 g) solution and treated with EtOAc. The organic phase was washed with three times with 100 ml of water and dried with Na_2SO_4, and EtOAc was evaporated in vacuum. The residue was precipitated with diethyl ether to remove excess benzyl alcohol and purified by flash chromatography using petroleum ether-EtOAc as an eluent yielding 50 mg (5%) of the solid material. ESI-MS: m/z = 423.23 (M + 1); ^{1}H NMR δ (ppm) ($CDCl_3$/CD_3OD): 4.93 (s, 2H), 6.62 (d, 2H), 6.65 (d, 2H); 6.90 (dd, 4H), 7.20 (t, 2H), 7.28 (t, 2H), 7.42 (s, 1H), 7.72 (t, 1H), 7.76 (t, 1H), 8.30 (d, 1H). ^{13}C NMR ($CDCl_3$/CD_3OD): 67.58, 103.59, 115.10, 128.11, 128.44, 128.48, 129.85, 130.09, 130.25, 130.30, 131.33, 132.72, 133.96, 134.01, 153.81, 157.16, 165.42. Analysis: calculated for $C_{27}H_{18}O_5 \cdot 0.75$ EtOAc: C, 73.76; H, 4.95; found: C, 74.09; H, 4.66.

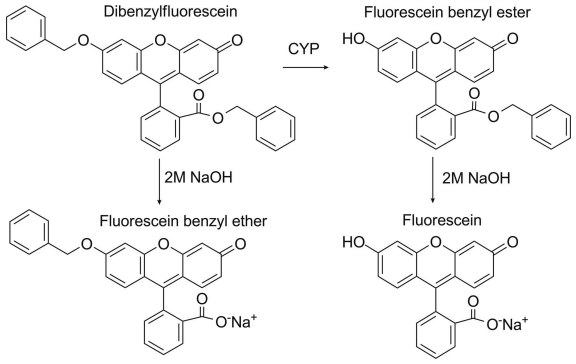

FIG. 1. Structures of a probe substrate, DBF, and fluorescent products. DBF is dealkylated by P450 to form a fluorescein benzyl ester, which is further hydrolyzed to fluorescein by NaOH (if present). Addition of 2 M NaOH causes also decomposition of DBF to fluorescein benzyl ether.

Synthesis of Fluorescein Benzyl Ether. Fluorescein methyl ester (Adamczyk et al., 1999) (1.75 g, 5.05 mmol), benzyl bromide (1.04 g, 6.06 mmol), and potassium carbonate (2.10 g, 15.2 mmol) in dimethylformamide (10 ml) were irradiated with microwaves at 75°C for 30 min (Fig. 1). Dimethylformamide was evaporated, and the residue was purified by flash chromatography using petroleum ether-EtOAc as an eluent yielding 520 mg (24%) of the solid material. ^{1}H NMR δ (ppm) ($CDCl_3$): 3.64 (s, 3H), 5.17 (s, 2H), 6.44 (d, 1H), 6.53 (dd, 1H), 6.80 (dd, 1H), 6.84 (d, 1H), 6.89 (d, 1H), 7.03 (d, 1H), 7.31 (d, 1H), 7.41 (m, 5H), 7.66 (t, 1H), 7.73 (t, 1H), 8.24 (d, 1H). The product (140 mg, 0.32 mmol) and lithium iodide (428 mg, 3.21 mmol) were dissolved in pyridine (5 ml) and were irradiated with microwaves at 120°C for 1 h. Pyridine was evaporated, and the residue was purified by flash chromatography using petroleum ether-EtOAc as an eluent yielding 60 mg (44%) of the solid material. ^{1}H NMR δ (ppm) ($CDCl_3$): 5.09 (s, 2H), 6.52 (dd, 1H), 6.63 (d, 1H), 6.67 (s, 1H) 6.68 (s, 1H), 6.71 (d, 1H), 6.84 (t, 1H), 7.16 (d, 1H), 7.39 (m, 5H), 7.61 (t, 1H), 7.66 (t, 1H), 8.01 (d, 1H). ESI-MS: m/z = 421.24 (M − 1); ^{13}C NMR ($CDCl_3$): 70.27, 101.85, 103.12, 111.21, 111.28, 112.22, 112.30, 123.98, 125.03, 126.73, 127.47, 128.19, 128.67, 129.09, 129.33, 129.71, 135.09, 136.23, 152.43, 152.48, 153.11, 157.73, 160.47, 169.93. Analysis: calculated for $C_{27}H_{17}O_5Li \cdot 0.1$ hexane: C, 75.86; H, 4.24; found: C, 75.97; H, 4.59.

P450-Catalyzed Metabolism of DBF and Effect of Base. CYP2C19-catalyzed biotransformation of DBF was analyzed by liquid chromatography (LC)-electrospray ionization-mass spectrometry (MS). Incubations were conducted in 500-μl volume in Eppendorf tubes using cDNA-expressed recombinant CYP2C19 enzyme. Incubation mixtures for enzyme-catalyzed samples contained 0.1 M Tris-HCl buffer (pH 7.4), 10 μM DBF, 15 pmol of CYP2C19 enzyme, and an NADPH-regenerating system (1.13 mM NADP, 12.5 mM isocitric acid, 56.33 mM KCl, 187.5 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 0.0125 mM $MnCl_2$, and 0.075 U/ml isocitrate dehydrogenase). Three kinds of blank samples were used: the first did not contain the P450 enzyme, the second lacked the NADPH-regenerating system, and the third lacked DBF. Otherwise the blanks were treated similarly to the enzyme-catalyzed samples. Each sample was assayed in duplicate. The samples were incubated for 45 min at 37°C. The reactions were terminated by rapid cooling to 4°C and after centrifugation, the supernatants were analyzed by LC-MS. Pure DBF, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein (all 10 μM) were used as standards and were analyzed in the absence and presence of 2 M

TABLE 1

Experimental conditions in CYP2C19 enzyme activity and inhibition assays in a 150-μl incubation volume

Condition	Value
Tris-HCl, pH 7.4	100 mM
Substrate (DBF) concentration	1.0 μM (equals K_m)
Enzyme (human recombinant CYP2C19)	1.5 pmol
NADPH-regenerating system	50 μl
Incubation time at 37°C	30–60 min
Excitation/emission wavelengths (nm)	485/535

NaOH. All samples were analyzed with a Finnigan LTQ mass spectrometer using positive electrospray ionization and full scan or MS/MS measurements. The compounds were separated using a 1200 high-performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany) equipped with a 50 × 2 mm Gemini C18 column (Phenomenex, Torrance, CA) with a gradient starting from 10% acetonitrile, 0.1% formic acid and ending with 90% acetonitrile, 0.1% formic acid in 10 min at a flow rate of 200 μl/min and injection volume of 5 μl. The column temperature was 30°C. The compounds were identified on the basis of their retention times, molecular weights, and MS/MS spectra.

Stability of Fluorescence Intensity. Stability of the fluorescence intensity of DBF, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein (all 1 μM) was characterized at different pH and temperatures (room temperature or 37°C). The buffers were either 100 mM Tris-HCl (pH 7.0 and 7.4) or 100 mM KPO_4 (pH 7.0 and 7.4). All samples were in a 150-μl total volume in duplicate in OptiPlate 96-well microplates, and the fluorescence intensity was measured with a Victor2 plate reader (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland) in a continuous mode at 1-min intervals for 90 min at excitation and emission wavelengths of 485 and 535 nm, respectively.

Prerequisites for Kinetic Assay. The initial prerequisites for a real-time kinetic assay were investigated using the endpoint assay procedure by determining whether enzyme activity could be detected without the use of 2 M NaOH. This was done by comparing signal/noise ratios between an enzyme-catalyzed sample and two blank (nonenzyme-catalyzed) samples. The experimental conditions were as described in Table 1. The reactions were initiated by addition of 50 μl of the NADPH-regenerating system after a 10-min preincubation at 37°C and were subsequently incubated for 30 min at 37°C in the dark. Blank I was similar to the control (enzyme-catalyzed) sample except that 110 μl of 2 M NaOH was added into the wells before addition of the NADPH-regenerating system. Blank II lacked the enzyme. After fluorescence was measured at the end of the 30-min incubation, 110 μl of 2 M NaOH was added into the wells containing control and blank II samples, and the fluorescence was measured again.

Real-Time Kinetic Assay. Real-time kinetic assays were conducted at 37°C in a Victor2 plate scanner using the experimental conditions in Table 1. Blank samples were treated similarly to the enzyme-catalyzed samples but in the absence of P450 enzyme. Reactions were initiated by addition of DBF (experiment A) or the NADPH-regenerating system (experiment B). Experiment C was conducted by measuring first the substrate in a Victor2 plate scanner for 15 min at 37°C, after which the prewarmed enzyme and the NADPH-regenerating system were added into the wells and immediately after that the actual reaction incubation, and fluorescence data acquisition was performed at 1-min intervals for 45 min. Each experiment was performed in duplicate. In addition, the linearity of the CYP2C19-catalyzed reaction with respect to enzyme concentration and incubation time was investigated by using varying amounts (0.125–2 pmol) of the enzyme.

TABLE 2

LC/MS characteristics of DBF, fluorescein, fluorescein benzyl ester, and fluorescein benzyl ether

Compound	Retention Time	MH^+	MS/MS Fragment Ions
	min	*m/z*	*m/z*
Dibenzylfluorescein	9.84	513.4	485,421,345,333
Fluorescein	7.25	333.2	305, 287 271
Fluorescein benzyl ester	7.28	423.3	361,345, 317
Fluorescein benzyl ether	9.75	423.3	395,377, 345

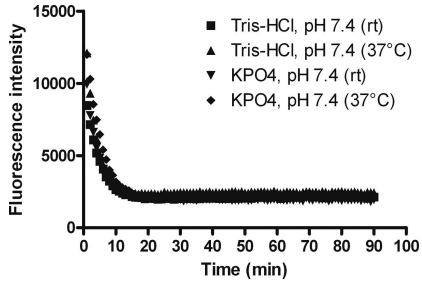

FIG. 2. Fluorescence intensity of 1 μM DBF as a function of time in different conditions.

Determination of IC_{50}. To compare determination of IC_{50} values between endpoint and kinetic assays, the IC_{50} value of the known CYP2C19 inhibitor tranylcypromine was assessed using four modified procedures: (A) traditional endpoint assay, (B) endpoint assay combined with initial measurement of the substrate for 15 min before the reaction initiation, and kinetic assays without (C) and with (D) substrate premeasurement. The reactions were initiated by addition of the enzyme and were incubated for 30 min at 37°C. In the endpoint assays, the reactions were terminated by addition of 110 μl of 2 M NaOH. In the kinetic assays, linear regions of the progress curves were used for the reaction velocity calculations. All IC_{50} values were determined as a mean value from duplicate determinations. Percentages of remaining P450 activity were plotted as a function of the logarithm of the molar concentration of tranylcypromine, and the curves were fitted to a sigmoid dose-response equation with Prism 4.0 software (GraphPad Software Inc., San Diego, CA).

Progress Curve Analysis. The procedure involving the 15-min initial measurement of substrate as shown in Fig. 3C was used in progress curve analysis. In these experiments, the enzyme is exposed simultaneously to the substrate and inactivator, and enzyme activity is monitored throughout the process (real-time kinetic assay). Progress curve experiments were performed by evaluating two known mechanism-based (time-dependent) inactivators, isoniazid and ticlopidine, and one known reversible (time-independent) inhibitor, tranylcypromine. Seven different concentrations of each test compound were used: 2.74 to 4000 μM for isoniazid, 0.14 to 100 μM for ticlopidine, and 0.14 to 100 μM for tranylcypromine. These concentrations were selected to ensure a wide range of inactivation across the 45-min time course. Isoniazid and ticlopidine were dissolved in water, and tranylcypromine was dissolved in acetonitrile and then further diluted with water. Consequently, the final solvent concentrations in the incubations did not exceed 2.2%. Controls were treated similarly but without the presence of inactivators. The fluorescence data obtained were analyzed to determine the key kinetic parameters and mechanistic information of the inactivation process.

Each progress curve was fitted by eq. 1 (Copeland, 2005), which contains terms for the initial and steady-state velocities (v_i and v_s) and for the rate constant for onset of inhibition (k_{obs}), i.e., conversion from the initial velocity phase to the steady-state velocity phase:

$$[\text{Product}] = v_s t + \frac{v_i - v_s}{k_{obs}}[1 - \exp(-k_{obs} t)] \quad (1)$$

TABLE 3

Signal/noise ratios of enzyme-catalyzed reactions

Blank[a]	Measurement	
	Immediately after Incubation	After the First Measurement[b]
	Fold difference between enzyme-catalyzed and blank samples	
Blank I	2.4	3.5
Blank II	11.9	17.9

[a] Blank I: as enzyme-catalyzed sample but 2 M NaOH added into samples before initiation of the reactions; blank II: as enzyme-catalyzed sample but in the absence of enzyme.
[b] 2 M NaOH was added into the wells that did not contain it.

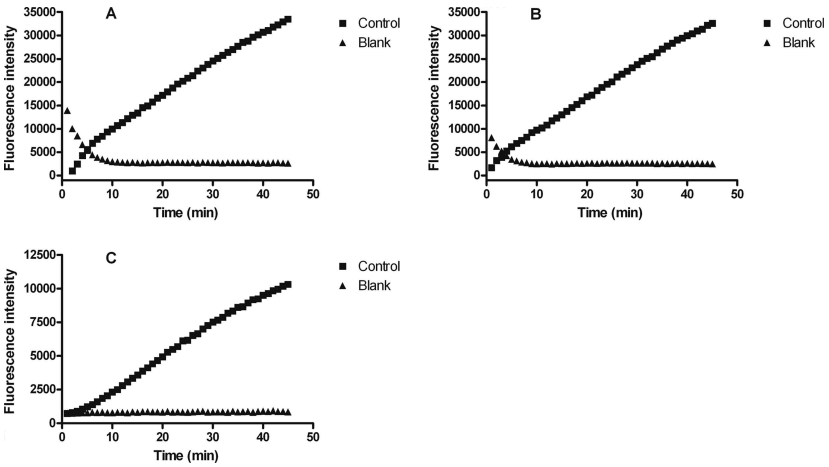

FIG. 3. Representative progress curves for CYP2C19 enzyme activity. A, reaction initiated by addition of substrate [r^2 value of the linear regression of the enzyme-catalyzed (control) reaction curve 0.987]. B, reaction initiated by addition of the NADPH-regenerating system ($r^2 = 0.997$). C, reaction initiated after a 15-min premeasurement of the substrate by addition of the NADPH-regenerating system and the enzyme ($r^2 = 0.997$).

K_{obs} values were then plotted against inactivator concentrations and fitted to eq. 2 (Fairman et al., 2007):

$$k_{obs} = \frac{k_{inact}[I]}{K_I(1 + S/K_m) + [I]} \quad (2)$$

where k_{inact} is the maximum rate of inactivation, $[I]$ is the concentration of inactivator, K_I is the inactivator concentration required for half-maximal inactivation, S is the substrate concentration, and K_m is the Henri-Michaelis-Menten constant.

Results

P450-Catalyzed Metabolism of DBF and Effect of Base. The CYP2C19-catalyzed biotransformation of DBF and effect of base were investigated by LC-MS. The retention times, MH^+ ions, and major MS/MS fragment ions for the standards were used for identification of the compounds (Table 2). Fluorescein benzyl ester was formed in the CYP2C19-catalyzed reaction as a major metabolite, and minor amounts of fluorescein benzyl ether were also detected. Fluorescein was not formed at the reaction pH 7.4. In the blank samples, fluorescein benzyl ester, fluorescein benzyl ether, and fluorescein were not formed from DBF. Addition of 2 M NaOH caused decomposition of DBF to fluorescein benzyl ether, and decomposition of fluorescein benzyl ester to fluorescein, whereas fluorescein and fluorescein benzyl ether remained unchanged in the presence of 2 M NaOH. The standard compounds remained unchanged in the absence of 2 M NaOH. The results are presented in Fig. 1.

Stability of Fluorescence Intensity. The fluorescence intensity of DBF decreased markedly during the first 10 min of the measurement until it reached a steady state (Fig. 2). The fluorescence intensity during the 90-min measurement was the same with the different buffers (Tris-HCl and KPO_4), pH conditions (pH 7.0 and 7.4), and temperatures (room temperature and 37°C). A similar quenching of fluorescence was observed for fluorescein benzyl ester and fluorescein benzyl ether but not for fluorescein (data not shown). At steady state, when the amounts of the fluorescence intensity of DBF were compared with the intensities of fluorescein benzyl ether, fluorescein benzyl ester, and fluorescein, these were 20-, 120-, and 200-fold higher than that of DBF, respectively.

Prerequisites for Kinetic Assay. The optimal conditions for the kinetic assay were examined by comparing fluorescence intensities between enzyme-catalyzed sample and two kinds of blank samples and by evaluating the effects of NaOH on fluorescence intensity. The results are summarized in Table 3. An acceptable signal/noise ratio was achieved during the 30-min incubation without addition of 2 M NaOH to the samples, because the fluorescence intensity was 12-fold higher in the enzyme-catalyzed samples than in blank II (no enzyme) sample. In contrast, the fluorescence intensity was only approximately 2- to 3-fold higher in the enzyme-catalyzed samples than in blank I (NaOH added before initiation of the reaction). One important finding was that the signal/noise ratio was approximately 5 times greater between the enzyme sample and blank II than between enzyme sample and blank I. Addition of 2 M NaOH at the end of incubation resulted in only a 1.5-fold increase in fluorescence intensity. The result showed that it is feasible to detect enzyme activity without the use of 2 M NaOH and that blank II is a better choice for assessing background noise than blank I.

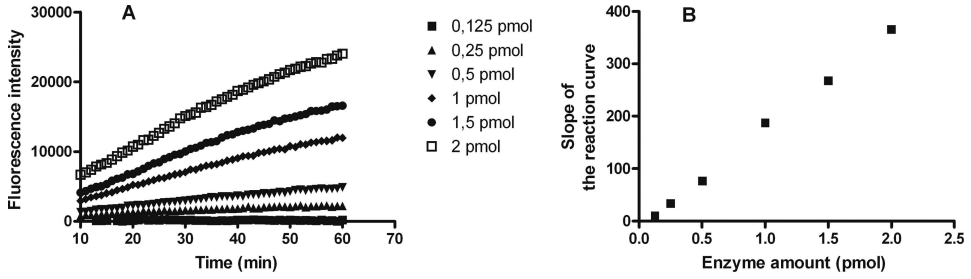

FIG. 4. A, progress curves of CYP2C19-mediated fluorescence formation with different amounts of the enzyme. B, slopes of the lines from A plotted as a function of enzyme amount (picomoles).

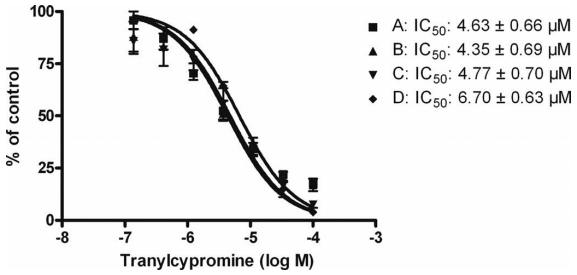

FIG. 5. IC_{50} values of tranylcypromine with endpoint (A and B) and kinetic (C and D) assays excluding (A and C) and including (B and D) a 15-min initial measurement of substrate.

Real-Time Kinetic Assay. The progress of the P450-mediated catalytic reaction can be observed from the kinetic readouts as shown in Fig. 3. The fluorescence intensity of DBF declined to the steady-state value during the 15-min initial measurement, allowing for detection of the linear reaction kinetics from the very beginning of the reaction (Fig. 3C).

The time course of the CYP2C19-catalyzed reaction with varying amount of the enzyme is presented in Fig. 4. Linear kinetics with respect to enzyme amount and incubation time was evident from the plots. Linear regression analysis of the plot of enzyme amount versus incubation time indicated that the best linearity was obtained with 1 to 2 pmol of the enzyme.

Determination of IC_{50}. The IC_{50} value of tranylcypromine was determined by the endpoint (experiments A and B) and the kinetic assays (experiments C and D). Inhibition of DBF metabolism by tranylcypromine is shown in Fig. 5.

Progress Curve Analysis. Progress curves for each of the inactivators are shown in Fig. 6. All reaction progress curves without inactivator displayed linear kinetics and confirmed that the measurements were made during the linear steady-state phase of the reaction. For isoniazid and ticlopidine, significant time- and concentration-dependent inactivation of CYP2C19 was observed, and their progress curves were fitted to eq. 1 to obtain estimates of k_{obs}, v_i, and v_s at several concentrations. Except for tranylcypromine, best-fit lines of the inactivators yielded v_s values of zero (Fig. 6).

The K_{obs} values were replotted against inactivator concentrations and fitted to eq. 2 (Fig. 7). The K_I and k_{inact} values determined are presented in Table 4. Tranylcypromine showed concentration- but not time-dependent inhibition determined by its linear progress curves at each concentration. Thus, it was analyzed by linear fitting of each concentration because the relative reaction velocity can be determined from the slope of a linear fit, and the IC_{50} value was determined via standard methodologies (4.72 ± 0.30 μM) and then converted to an absolute inhibition constant (K_i 2.36 μM) for substrate affinity and concentration using the Cheng-Prusoff equation, where $K_i = IC_{50}/(1 + [S]/K_m)$. All results represent the mean of duplicate determinations.

Discussion

In the present study, we describe development of a simple, direct, and informative fluorometric method for the assessment of CYP2C19 enzyme inactivation kinetics. Well known inactivators were used to test the method. Isoniazid and ticlopidine were chosen because clinically relevant interactions between them and substrates of CYP2C19 have been reported, and they are known to act via MBI (Donahue et al., 1997; Tateishi et al., 1999; Nishimura et al., 2003; Richter et al., 2004; Kalgutkar et al., 2007; Venkatakrishnan and Obach, 2007). The selected competitive inhibitor, tranylcypromine, is commonly used as a positive control compound for CYPC19 inhibition studies (BD Gentest, A high throughput method for measuring cytochrome P450 inhibition, 2000, http://www.bdj.co.jp/gentest/1f3pro00000sf5lj-att/P450-InhibitorScreeningTechBulletin-Ver4.2-2000-09.pdf) (Lin et al., 2007).

The data show that 2 M NaOH is not required for enzyme activity determinations using DBF as the probe substrate because the fluores-

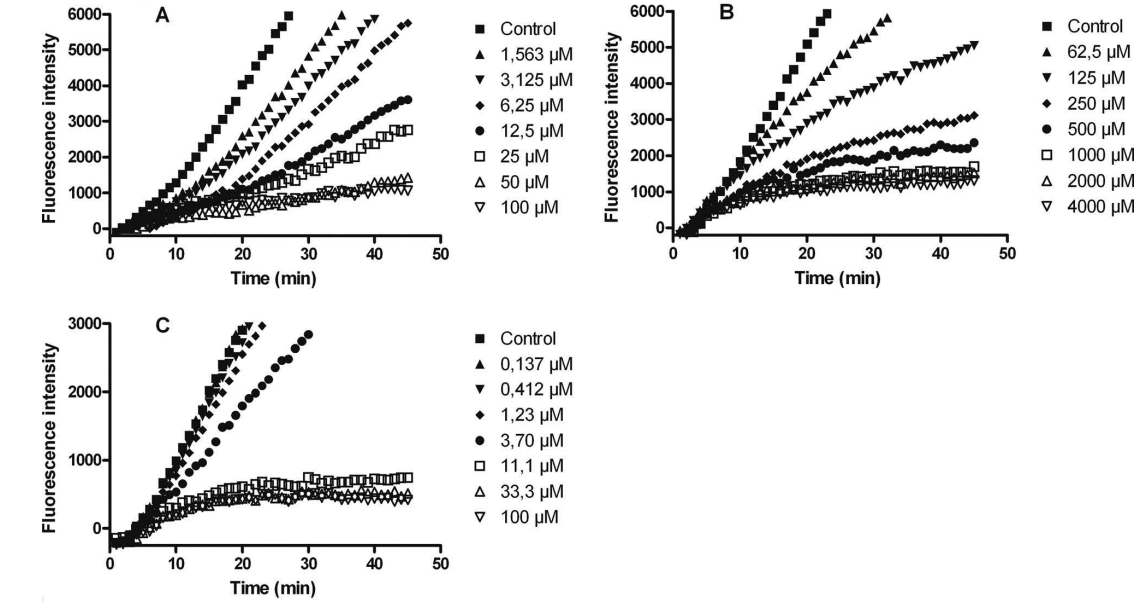

FIG. 6. Representative progress curves of CYP2C19 inactivation. Tranylcypromine (A) reveals no time-dependent inhibition because the curves indicate linear best fits. The known mechanism-based inactivators, isoniazid (B) and ticlopidine (C), exhibit time-dependent inactivation.

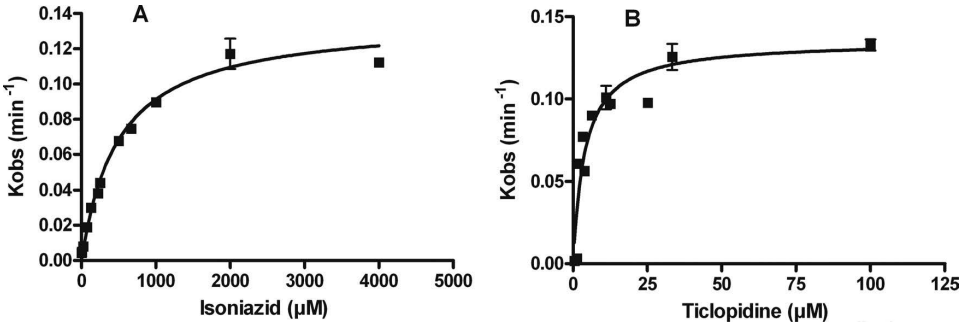

FIG. 7. Plots of k_{obs} against inactivator concentration for isoniazid (A) and ticlopidine (B) indicate a two-step inactivation mechanism due to a saturable step before inactivation.

cence intensity of the metabolite formed (fluorescein benzyl ester) is 120-fold higher than that of DBF. The transformation of fluorescein benzyl ester to fluorescein in the presence of 2 M NaOH is consistent with the reports of its use in the endpoint assays (Miller et al., 2001; Hong et al., 2008). However, it is not desirable that at the same time DBF is transformed to fluorescein benzyl ether in the presence of 2 M NaOH, which also increases the background fluorescence intensity and causes significant deterioration in the sensitivity, i.e., signal/noise ratio.

Therefore, CYP2C19 enzyme activity can be followed in real time using DBF as the substrate. The product formation is linear from the very beginning of the reaction, and the IC_{50} values calculated from the progress curves are comparable with the results obtained with the traditional endpoint assay and correlated well with literature data (a range of 1.9 to 9 μM) (BD Gentest, A high throughput method for measuring cytochrome P450 inhibition, 2000 (Dierks et al., 2001; Lin et al., 2007). A kinetic assay provides the most reliable means of accurately determining reaction velocity from the slope of a plot of signal versus time, i.e., it is purely a result of the enzyme activity itself. Based on these experiments, the observed spontaneous quenching of DBF fluorescence is due to some fluorometric phenomenon instead of decomposition of the compound. The other related compounds possess higher fluorescence intensities than DBF.

The full progress curve of an enzymatic reaction contains an abundance of valuable kinetic information, allowing investigation of both reversible and irreversible components of the reaction mechanisms, and thus provides more information in one experiment. All irreversible enzyme inactivators display slow binding kinetics in progress curve analysis, and the slow onset of inhibition is best studied by following progress curves. Time-dependent inactivation converts the linear progress curve seen in the absence of the inactivator into a curvilinear function so that the degree of inhibition at a fixed concentration of compound will vary over time. Thus, the enzyme reaction progress curve will be nonlinear and reflect two distinct velocities for the reaction (Copeland, 2005). This behavior was seen with the known time-dependent inactivators, isoniazid and ticlopidine, but not with the known time-independent inhibitor, tranylcypromine.

For time-dependent irreversible/quasi-irreversible inactivators, the value of k_{obs} is generally expected to increase over a certain range of inactivator concentrations and then to undergo saturation at higher concentrations (Kalgutkar et al., 2007). The first step involves reversible binding of the inactivator to the enzyme, often under rapid equilibrium conditions. For mechanism-based inactivators, the second step involves some bioactivation/chemistry of the covalent bond formation or transformation into metabolic intermediate products that coordinate tightly to the heme iron atom of P450 enzyme. This behavior (saturation) was also seen with isoniazid and ticlopidine in this study, pointing to a two-step inactivation mechanism.

All mechanism-based inactivators are competitive with the normal substrate of the enzyme, because they rely on the catalytic mechanism of the enzyme active site. The presence of substrate can hinder the access of the inactivator to the enzyme, i.e., the substrate has to be at a relatively low concentration (K_m) so that it does not completely block the enzyme inactivation process (Ghanbari et al., 2006). For that reason, the probe substrate at the concentration corresponding to its measured apparent K_m (data not shown) was used in the experiments, and this was taken into account when kinetic constants of the inactivation were determined. The inactivation kinetic constants (K_I and k_{inact}) determined for isoniazid and ticlopidine correlated with published data (Wen et al., 2002; Nishimura et al., 2003; Polasek et al., 2006; Kalgutkar et al., 2007; Venkatakrishnan and Obach, 2007; Nishiya et al., 2009).

This progress curve analysis methodology allows estimates of the initial binding equilibrium as reflected in the magnitude of reduction of v_i by the inactivator against respective control, as described by Fairman et al. (2007). In our study, v_i was plotted against the initial

TABLE 4

Inactivation kinetic constants for isoniazid and ticlopidine

Inactivator	Determined		Published Data		Reference
	K_I	k_{inact}	K_I	k_{inact}	
	μM	min^{-1}	μM	min^{-1}	
Isoniazid	250.5 ± 34	0.137 ± 0.006	112	0.090	Wen et al. (2002)
			255	0.020	Nishimura et al. (2003)
			79.3	0.039	Polasek et al. (2006)
Ticlopidine	1.96 ± 0.5	0.135 ± 0.009	1.65	0.192	Venkatakrishnan and Obach (2007)
			4.30	0.097	Kalgutkar et al. (2007)
			3.32	0.074	Nishiya et al. (2009)

inactivator concentration but no significant inhibition was seen across the concentration range (<40–50% inhibition at any concentration tested) so that no estimation of K_i could be made.

In conclusion, time-dependent inhibition is one of the major distinguishing features between reversible and irreversible/quasi-irreversible inhibition. It thus provides a useful screening approach for identifying potential mechanism-based inactivators in early drug interaction studies for pharmaceutical agents under development. This finding is especially important because it has become widely recognized that detection and amelioration of time-dependent inactivation is a crucial aspect in drug interaction optimization for novel compounds (Polasek and Miners, 2007; Fowler and Zhang, 2008). DBF characterization, kinetic assay, and the progress curve analysis approach together offer a new response and improvement for the current challenge. The present work has shown that the widely used P450 substrate, DBF, is suitable for use in a progress curve analysis approach; i.e., this is a rapid and reliable method that can be used as an initial screen to help identify compounds that require more detailed investigations. However, this approach will require further evaluation with a broader set of P450 enzymes and inactivators before it is fully exploitable.

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Authorship Contributions

Participated in research design: Salminen, Venäläinen, and Raunio.

Conducted experiments: Salminen and Auriola.

Contributed new reagents or analytic tools: Leppänen.

Performed data analysis: Salminen.

Wrote or contributed to the writing of the manuscript: Salminen, Venäläinen, Pasanen, Auriola, Juvonen, and Raunio.

References

Adamczyk M, Grote J, and Moore JA (1999) Chemoenzymatic synthesis of 3′-*O*-(carboxyalkyl)fluorescein labels. *Bioconjug Chem* **10:**544–547.

Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, et al. (2003) The conduct of in vitro and in vivo drug-drug interaction studies: a PhRMA perspective. *J Clin Pharmacol* **43:**443–469.

Copeland RA (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery. a Guide for Medicinal Chemists and Pharmacologists,* John Wiley & Sons, Inc., Hoboken, NJ.

Crespi CL, Miller VP, and Penman BW (1997) Microtiter plate assays for inhibition of human, drug-metabolizing cytochromes P450. *Anal Biochem* **248:**188–190.

Crespi CL and Stresser DM (2000) Fluorometric screening for metabolism-based drug-drug interactions. *J Pharmacol Toxicol Methods* **44:**325–331.

Dierks EA, Stams KR, Lim HK, Cornelius G, Zhang H, and Ball SE (2001) A method for the simultaneous evaluation of the activities of seven major human drug-metabolizing cytochrome P450s using an in vitro cocktail of probe substrates and fast gradient liquid chromatography tandem mass spectrometry. *Drug Metab Dispos* **29:**23–29.

Donahue SR, Flockhart DA, Abernethy DR, and Ko JW (1997) Ticlopidine inhibition of phenytoin metabolism mediated by potent inhibition of CYP2C19. *Clin Pharmacol Ther* **62:**572–577.

Fairman DA, Collins C, and Chapple S (2007) Progress curve analysis of CYP1A2 inhibition: a more informative approach to the assessment of mechanism-based inactivation? *Drug Metab Dispos* **35:**2159–2165.

Fowler S and Zhang H (2008) In vitro evaluation of reversible and irreversible cytochrome P450 inhibition: current status on methodologies and their utility for predicting drug-drug interactions. *AAPS J* **10:**410–424.

Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT, and Rostami-Hodjegan A (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Curr Drug Metab* **7:**315–334.

Grime KH, Bird J, Ferguson D, and Riley RJ (2009) Mechanism-based inhibition of cytochrome P450 enzymes: an evaluation of early decision making in vitro approaches and drug-drug interaction prediction methods. *Eur J Pharm Sci* **36:**175–191.

Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, et al. (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the Pharmaceutical Research and Manufacturers of America. *Drug Metab Dispos* **37:**1355–1370.

Hong Y, Cho M, Yuan YC, and Chen S (2008) Molecular basis for the interaction of four different classes of substrates and inhibitors with human aromatase. *Biochem Pharmacol* **75:**1161–1169.

Kalgutkar AS, Obach RS, and Maurer TS (2007) Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. *Curr Drug Metab* **8:**407–447.

Lin T, Pan K, Mordenti J, and Pan L (2007) In vitro assessment of cytochrome P450 inhibition: strategies for increasing LC/MS-based assay throughput using a one-point IC_{50} method and multiplexing high-performance liquid chromatography. *J Pharm Sci* **96:**2485–2493.

Miller VP, Stresser D, Crespi CL, and Charles L (2001), inventors; Gentest Corp, assignee.The use of fluorescein aryl ethers in high throughput cytochrome P450 inhibition assays. World patent WO0114361 (A1). 2001 March 1.

Nishimura Y, Kurata N, Iwase M, and Yasuhara H (2003) Mechanism-based inactivation of human CYP1A2 and CYP2C19-mediated metabolism by isoniazid. *Drug Metab Rev* **35:**50.

Nishiya Y, Hagihara K, Kurihara A, Okudaira N, Farid NA, Okazaki O, and Ikeda T (2009) Comparison of mechanism-based inhibition of human cytochrome P450 2C19 by ticlopidine, clopidogrel, and prasugrel. *Xenobiotica* **39:**836–843.

Obach RS (2009) Predicting drug-drug interactions from in vitro drug metabolism data: challenges and recent advances. *Curr Opin Drug Discov Devel* **12:**81–89.

Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J, and Raunio H (2008) Inhibition and induction of human cytochrome P450 enzymes: current status. *Arch Toxicol* **82:**667–715.

Polasek TM, Elliot DJ, Somogyi AA, Gillam EM, Lewis BC, and Miners JO (2006) An evaluation of potential mechanism-based inactivation of human drug metabolizing cytochromes P450 by monoamine oxidase inhibitors, including isoniazid. *Br J Clin Pharmacol* **61:**570–584.

Polasek TM and Miners JO (2007) In vitro approaches to investigate mechanism-based inactivation of CYP enzymes. *Expert Opin Drug Metab Toxicol* **3:**321–329.

Richter T, Mürdter TE, Heinkele G, Pleiss J, Tatzel S, Schwab M, Eichelbaum M, and Zanger UM (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J Pharmacol Exp Ther* **308:**189–197.

Riley RJ, Grime K, and Weaver R (2007) Time-dependent CYP inhibition. *Expert Opin Drug Metab Toxicol* **3:**51–66.

Tateishi T, Kumai T, Watanabe M, Nakura H, Tanaka M, and Kobayashi S (1999) Ticlopidine decreases the in vivo activity of CYP2C19 as measured by omeprazole metabolism. *Br J Clin Pharmacol* **47:**454–457.

Wallentin L (2009) $P2Y_{12}$ inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. *Eur Heart J* **30:**1964–1977.

Wen X, Wang JS, Neuvonen PJ, and Backman JT (2002) Isoniazid is a mechanism-based inhibitor of cytochrome P450 1A2, 2A6, 2C19 and 3A4 isoforms in human liver microsomes. *Eur J Clin Pharmacol* **57:**799–804.

Venkatakrishnan K and Obach RS (2007) Drug-drug interactions via mechanism-based cytochrome P450 inactivation: points to consider for risk assessment from in vitro data and clinical pharmacologic evaluation. *Curr Drug Metab* **8:**449–462.

Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4:**825–833.

Zhou ZW and Zhou SF (2009) Application of mechanism-based CYP inhibition for predicting drug-drug interactions. *Expert Opin Drug Metab Toxicol* **5:**579–605.

Address correspondence to: Kaisa A. Salminen, School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland. E-mail: kaisa.salminen@uef.fi

III

CYP2C19 progress curve analysis and mechanism-based inactivation by three methylenedioxyphenyl compounds

Salminen KA, Meyer A, Imming P and Raunio H

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CYP2C19 Progress Curve Analysis and Mechanism-Based Inactivation by Three Methylenedioxyphenyl Compounds

Kaisa A. Salminen, Achim Meyer, Peter Imming, and Hannu Raunio

School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland (K.A.S., H.R.); and Institut für Pharmazie, Abteilung Pharmazeutische Chemie, Martin-Luther-Universität Halle-Wittenberg, Halle, Germany (A.M., P.I.)

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ABSTRACT:

Several in vitro criteria were used to assess whether three methylenedioxyphenyl (MDP) compounds, the isoquinoline alkaloids bulbocapnine, canadine, and protopine, are mechanism-based inactivators of CYP2C19. The recently reported fluorometric CYP2C19 progress curve analysis approach was applied first to determine whether these alkaloids demonstrate time-dependent inhibition. In this experiment, bulbocapnine, canadine, and protopine displayed time dependence and saturation in their inactivation kinetics with K_I and k_{inact} values of 72.4 ± 14.7 μM and 0.38 ± 0.036 min^{-1}, 2.1 ± 0.63 μM and 0.18 ± 0.015 min^{-1}, and 7.1 ± 2.3 μM and 0.24 ± 0.021 min^{-1}, respectively. Additional studies were performed to determine whether other specific criteria for mechanism-based inactivation were fulfilled: NADPH dependence, irreversibility, and involvement of a catalytic step in the enzyme inactivation. CYP2C19 activity was not significantly restored by dialysis when it had been inactivated by the alkaloids in the presence of a NADPH-regenerating system, and a metabolic-intermediate complex-associated increase in absorbance at approximately 455 nm was observed. In conclusion, the CYP2C19 progress curve analysis method revealed time-dependent inhibition by these alkaloids, and additional experiments confirmed its quasi-irreversible nature. This study revealed that the CYP2C19 progress curve analysis method is useful for identifying novel mechanism-based inactivators and yields a wealth of information in one run. The alkaloids bulbocapnine, canadine, and protopine, present in herbal medicines, are new mechanism-based inactivators and the first MDP compounds exhibiting quasi-irreversible inactivation of CYP2C19.

Introduction

Inhibition of cytochrome P450 (P450) enzymes is a major mechanism for metabolism-based drug interactions. Today many clinically relevant drug interactions are known to be due to impairment of metabolic clearance via mechanism-based inactivation (MBI) of various P450 forms. Mechanism-based inactivators are generally defined as compounds that are biotransformed by the catalytic mechanism of the enzyme into a species that can form heme or protein adducts or a metabolic inhibitory complex (MIC). MBI is generally of greater concern than reversible inhibition because it can result in a more profound and prolonged effect than might be anticipated from the therapeutic dose or exposure. There are some dramatic examples of this type of drug interaction, for example, the potent inhibition of CYP3A4 by the calcium channel blocker mibefradil (Wienkers and Heath, 2005; Ghanbari et al., 2006; Venkatakrishnan and Obach, 2007).

CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 together metabolize more than 90% of known drugs (Wienkers and Heath, 2005; Guengerich, 2008). CYP2C19 plays an important role in the metabolism of many drugs; most of the proton pump inhibitors and some antidepressant, antipsychotic, antiepileptic, and antiplatelet drugs are metabolized by CYP2C19 (Rendic, 2002; Pelkonen et al., 2008; Wang et al., 2009). In comparison with the other major P450 forms, relatively little has been published concerning CYP2C19 inhibitors, although screening during the early phases of drug metabolism studies with new chemical entities generally relies on the availability of highly potent and selective "diagnostic" inhibitors of individual P450 forms (Pelkonen et al., 2008; Khojasteh et al., 2011). CYP2C19 is also a rare exception among the human principal drug-metabolizing cytochrome P450 enzymes in that its crystal structure is not yet available.

In vitro kinetic assessment and prediction of drug interactions attributable to reversible inhibition (e.g., competitive) and MBI rely on operationally and mechanistically distinct approaches. Time-dependent inhibition (TDI) is one of the major features that distinguish MBI from reversible inhibition. The current guidelines on investigation of drug interactions by the U.S. Food and Drug Administration (FDA) (Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, 2006; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) and European Medicines Agency (Guideline on the Investigation of Drug Interactions, 2010; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/05/WC500090112.pdf) recommend testing of drug candidates also for mechanism-based inactivator properties. A mul-

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ABBREVIATIONS: P450, cytochrome P450; MBI, mechanism-based inactivation; MIC, metabolic-intermediate complex; TDI, time-dependent inhibition; FDA, U.S. Food and Drug Administration; MDP, methylenedioxyphenyl; DBF, dibenzylfluorescein.

titiered approach was recently recommended, i.e., abbreviated assays to determine at first whether new chemical entities demonstrate TDI, followed by more thorough inactivation studies for positive compounds (Grimm et al., 2009). However, development of effective screening methods for analyzing MBI is still at an early stage. The ability to use in vitro human P450 TDI data for in vivo predictions should be viewed as a prerequisite for generating the data. There are two important terms in such activity predictions, i.e., the maximal inactivation rate constant (k_{inact}) and the inactivator concentration required for half-maximal inactivation (K_I). However, first-line screening assays typically involve characterization of an IC_{50} value or a time-dependent shift in IC_{50} (Venkatakrishnan and Obach, 2007; Grime et al., 2009).

Compounds containing a methylenedioxyphenyl (MDP) or 1,3-benzodioxole moiety can inactivate P450 enzymes via MIC formation with the enzyme (Casida, 1970; Franklin, 1971). The MDP moiety is found in several drugs, pesticides, and naturally occurring compounds (Fukuto et al., 1991; Murray, 2000; Fontana et al., 2005; Kalgutkar et al., 2007). The phosphodiesterase-5 inhibitor tadalafil and the selective serotonin reuptake inhibitor paroxetine are examples of drugs that contain MDP. These two drugs are known to form MIC with human CYP3A4 and CYP2D6, respectively, resulting in mechanism-based inactivation of these enzymes (Bertelsen et al., 2003; Ring et al., 2005; Kalgutkar et al., 2007).

We reported recently a novel fluorometric progress curve analysis approach for rapid identification of TDI of CYP2C19 (Salminen et al., 2011a). The isoquinoline alkaloids bulbocapnine, canadine, and protopine contain the MDP moiety and inhibit CYP2C19 activity (Salminen et al., 2011b). The purpose of this study was to investigate the inhibition mechanism of CYP2C19 by these herbal medicine alkaloids and to apply the progress curve analysis method for evaluating the potential MBI mode of inhibition. This method was shown to yield a wealth of information in one run, and additional experiments demonstrated the quasi-irreversible nature of inhibition by these alkaloids.

Materials and Methods

Materials. The origin of the isoquinoline alkaloids tested was described previously (Salminen et al., 2011b): protopine was isolated from *Bocconia cordata* Willd., bulbocapnine was isolated from *Corydalis cava* L., and canadine is a semisynthetic compound from berberine. Isoniazid and tranylcypromine were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Dibenzylfluorescein (DBF) (purity >99%) and cDNA-expressed human wild-type CYP2C19 (Supersomes) (with cytochrome b_5) were purchased from BD Biosciences Discovery Labware (Bedford, MA).

Progress Curve Analysis. The progress curve analyses were performed as described recently (Salminen et al., 2011a). This analysis uses an "all-in" approach in which the enzyme (1.5 pmol of CYP2C19) is exposed simultaneously to the probe substrate dibenzylfluorescein (1 μM DBF), inhibitor or the vehicle, and the NADPH-regenerating system in a 150-μl total volume in microplate wells. The enzyme activity is monitored by measuring the fluorescence intensity in a continuous mode at 1-min intervals for 45 min at excitation and emission wavelengths of 485 and 535 nm, respectively. DBF at 1 μM and seven different concentrations of each alkaloid were used: 0.274 to 200 μM for protopine, 6.25 to 400 μM for bulbocapnine, and 0.082 to 60 μM for canadine. These inhibitor concentrations were selected to ensure a wide range of inactivation across the 45-min time course. Controls were treated similarly but without addition of inhibitor. The fluorescence data obtained were analyzed to determine the key kinetic parameters (k_{obs}, k_{inact}, and K_I) and mechanistic information on the inactivation process. All results represent the mean of duplicate determinations.

Irreversibility of Inactivation. To assess whether removal of unbound inhibitor from the enzyme solution would reverse the CYP2C19 inactivation, samples were dialyzed after inactivation. A single concentration of each inhibitor (causing >90% inactivation on the basis of the progress curve analysis) was incubated with CYP2C19 enzyme in the presence and absence of the NADPH-regenerating system at 37°C for 30 min before dialysis. Controls were treated similarly but with no inhibitors included. The incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), the inhibitor (200 μM bulbocapnine, 100 μM canadine, 150 μM protopine, 2 mM isoniazid, or 150 μM tranylcypromine), 0.1 μM CYP2C19, and the NADPH-regenerating system or the vehicle. After the 30-min incubation, the samples were dialyzed in Slide-A-Lyzer mini-dialysis units (molecular weight cutoff 3500; Thermo Fisher Scientific, Waltham, MA) against 2.0 liters of 0.1 M potassium phosphate buffer, pH 7.4 (four 500-ml treatments for 2 h each) for 10 to 12 h at 4°C. Equivalents before the dialysis and from the dialyzed samples were collected into 96-well plates for enzyme activity measurements. The reactions were initiated by addition of the NADPH-regenerating system and the substrate

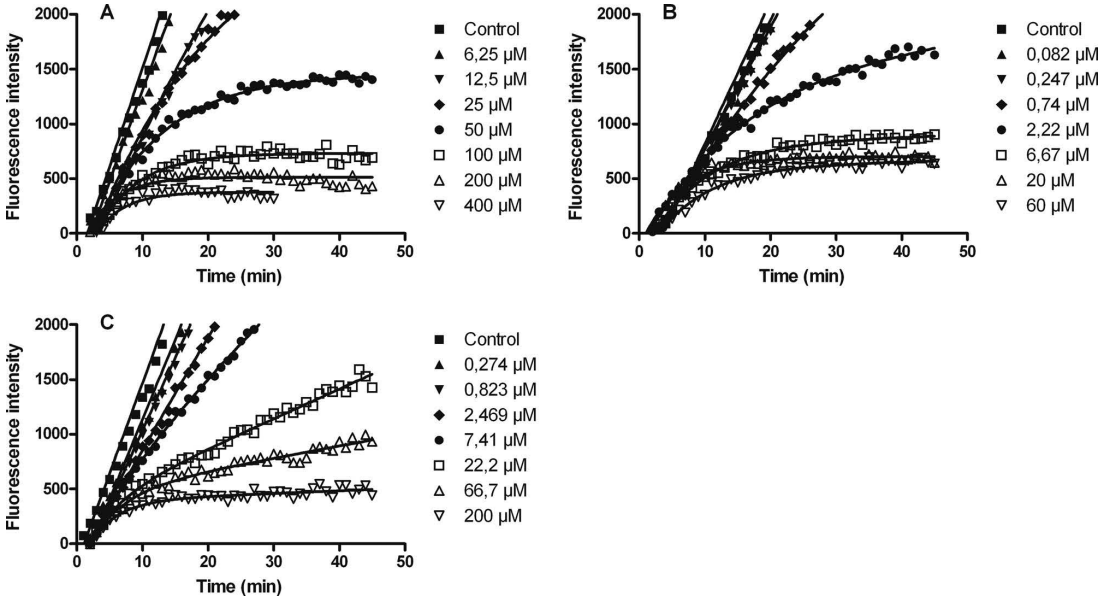


FIG. 1. Representative progress curves of CYP2C19 inactivation by bulbocapnine (A), canadine (B), and protopine (C), indicating time-dependent inhibition.

(1 μM), and the reactions were measured at 1-min intervals for 60 min. The known mechanism-based inactivator, isoniazid, was used as a positive control, and the known reversible inhibitor, tranylcypromine, was used as a negative control.

Spectroscopic Determination of MIC Formation. CYP2C19 Supersomes were used to characterize MIC formation associated with the metabolism of bulbocapnine (166.7 μM), canadine (100 μM), protopine (100 μM), and tranylcypromine (100 μM). Tranylcypromine was used as a negative control because it is a known reversible inhibitor. The incubation mixtures contained 0.1 M potassium phosphate buffer (pH 7.4), 0.13 μM CYP2C19 enzyme, the inactivator, and the NADPH-regenerating system, whereas the reference sample contained the vehicle used to dissolve the inhibitor. All MIC formation experiments were initiated by the addition of the NADPH-regenerating system and maintained at 37°C for 15 min. MIC formation was observed with an EnVision 2104 multilabel plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA) by scanning from 400 to 500 nm to monitor changes in the absorbance spectra. A similar assay procedure but in the absence of the NADPH-regenerating system was also performed. All experiments were performed in duplicate. Data were analyzed using Prism 5.0 software (GraphPad Software Inc., San Diego, CA).

Determination of IC_{50}. IC_{50} values for bulbocapnine, canadine, and protopine were determined essentially as described previously (Salminen et al., 2011a,b) using the kinetic assay procedure with seven different concentrations of each inhibitor. The mixture containing the inhibitor, enzyme, and NADPH-regenerating system was first preincubated for 30 min at 37°C, after which the reactions were initiated by addition of the substrate, and enzyme activity was monitored at 1-min intervals for 30 min. All IC_{50} values were determined as means from duplicate determinations. Linear regions of the progress curves were used for the reaction velocity calculations. Percentages of remaining enzyme activity were plotted as a function of the logarithm of the molar concentration of inhibitor, and the curves were fitted to a sigmoid dose-response equation with Prism 4.0 software (GraphPad Software Inc.).

Results

Progress Curve Analysis. The reaction progress curves for each of the alkaloids are shown in Fig. 1. All progress curves without the inactivator displayed linear kinetics and confirmed that the measurements were being made during the linear steady-state phase of the reaction. Significant time- and concentration-dependent inactivation of CYP2C19 was observed for all three alkaloids as deduced from the curvilinear function of the curves (Fig. 1). Thus, data analysis was performed as described previously (Salminen et al., 2011a) to obtain estimates of k_{obs}, v_i, and v_s values (Fig. 1) and of K_I and k_{inact} values (Fig. 2). The plots in Fig. 2 reveal a two-step binding and inactivation mechanism as seen by the saturation at higher concentrations. The K_I and k_{inact} values determined are presented in Table 1.

Irreversibility of Inactivation. The alkaloids investigated displayed characteristics in the dialysis experiment similar to those of isoniazid. The activity of CYP2C19, which had been inactivated by bulbocapnine, canadine, protopine, or isoniazid was not restored by dialysis or recovered only slightly when the NADPH-regenerating system was included in the inactivation mixture. In contrast, a dramatic recovery of enzyme activity could be achieved in the absence of the NADPH-regenerating system in the inactivation mixture. The activity of CYP2C19 inactivated by tranylcypromine was restored by dialysis in the presence and absence of the NADPH-regenerating

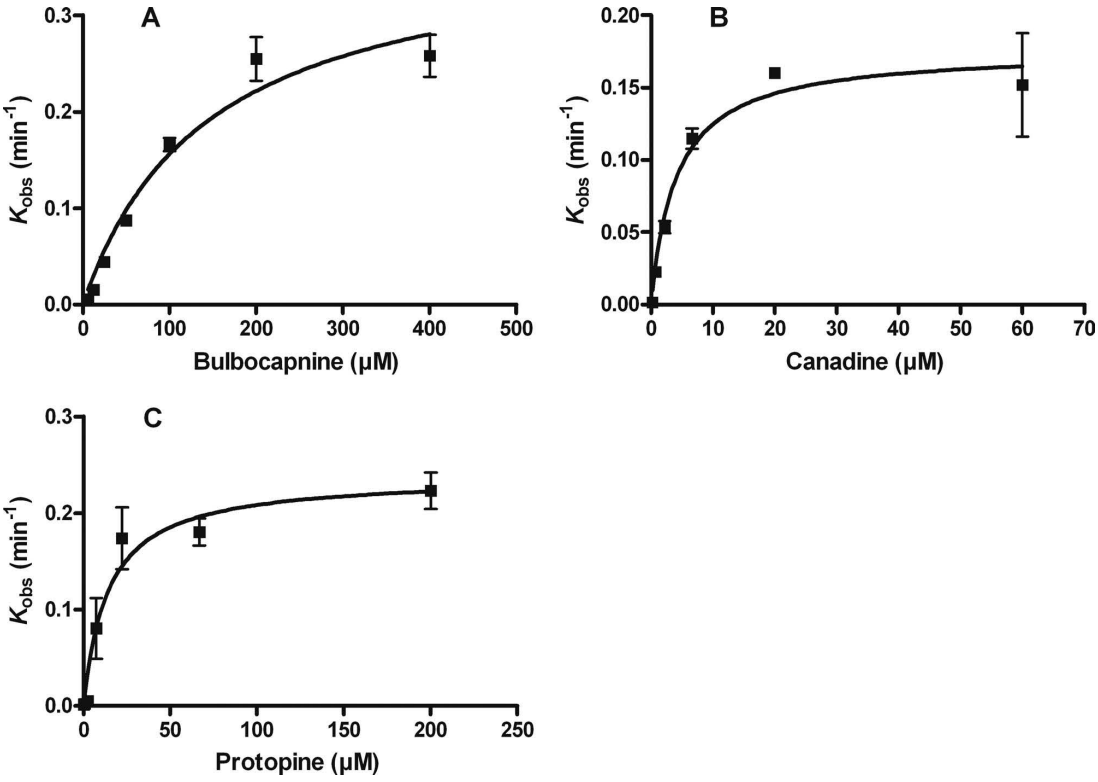
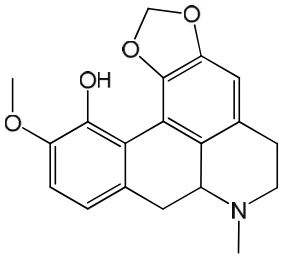
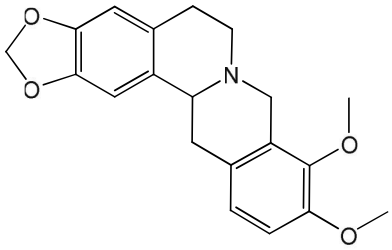
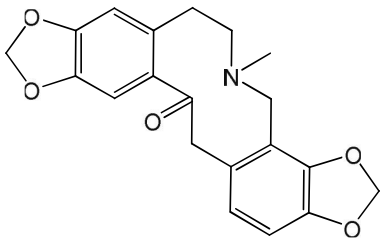


FIG. 2. Plots of k_{obs} against inactivator concentrations for bulbocapnine (A), canadine (B), and protopine (C), indicating a two-step binding and inactivation mechanism due to a saturable step before inactivation.

TABLE 1

Inactivation kinetic constants for the alkaloids

Alkaloid		Determined	
		K_I	k_{inact}
		μM	min^{-1}
Bulbocapnine		72.4 ± 14.7	0.38 ± 0.036
Canadine		2.1 ± 0.63	0.18 ± 0.015
Protopine		7.1 ± 2.3	0.24 ± 0.021

system in the inactivation mixture. The results are summarized in Table 2.

Spectroscopic Determination of MIC Formation. The MIC-associated increase in absorbance at approximately 455 nm was observed in the case of canadine and protopine and at approximately 450 nm in the case of bulbocapnine, but no MIC-associated increase in absorbance was observed for tranylcypromine. None of the compounds produced the characteristic peak at ~455 nm in the absence of the NADPH-regenerating system (Fig. 3).

TABLE 2

CYP2C19 activity before and after dialysis

Compound	CYP2C19 Inactivation (30 min)	Inhibition		
		Before Dialysis	After Dialysis	
			6 H	12 H
		%		
Isoniazid	+ NADPH[a]	93.5	89.6	85.2
	− NADPH[a]	75.4	52.0	27.8
Protopine	+ NADPH[a]	90.7	91.4 (4 h)	94.1 (10 h)
	− NADPH[a]	87.3	81.9 (4 h)	52.6 (10 h)
Bulbocapnine	+ NADPH[a]	91.9	89.1	86.0
	− NADPH[a]	87.3	27.9	23.4
Canadine	+ NADPH[a]	91.7	87.5	80.0
	− NADPH[a]	88.3	86.3	49.6
Tranylcypromine	+ NADPH[a]	73.8	30.6	0
	− NADPH[a]	75.3	27.2	0

[a] The NADPH-regenerating system.

Determination of IC_{50}. The IC_{50} values of bulbocapnine, canadine, and protopine were assessed using the standard 30-min preincubation as recommended by the FDA (Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling,, 2006, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) concerning TDI, and were compared with the inhibition potency category screened earlier without actual preincubation (Salminen et al., 2011b). The inhibition potency categories of bulbocapnine and canadine changed from weak (17.3 μM) to moderately potent (8.4 μM) and from moderately potent (2.2 μM) to potent (0.29 μM), respectively (Table 3). The IC_{50} value determination with the 30-min preincubation did not change the inhibition potency category of protopine (2.6 versus 1.8 μM). In addition, the IC_{50} values of bulbocapnine, canadine, and protopine were also found to increase according to increasing substrate (DBF) concentration, indicating substrate protection against inhibition (data not shown).

Discussion

The present work demonstrated that the recently reported (Salminen et al., 2011a) progress curve analysis is a useful screening

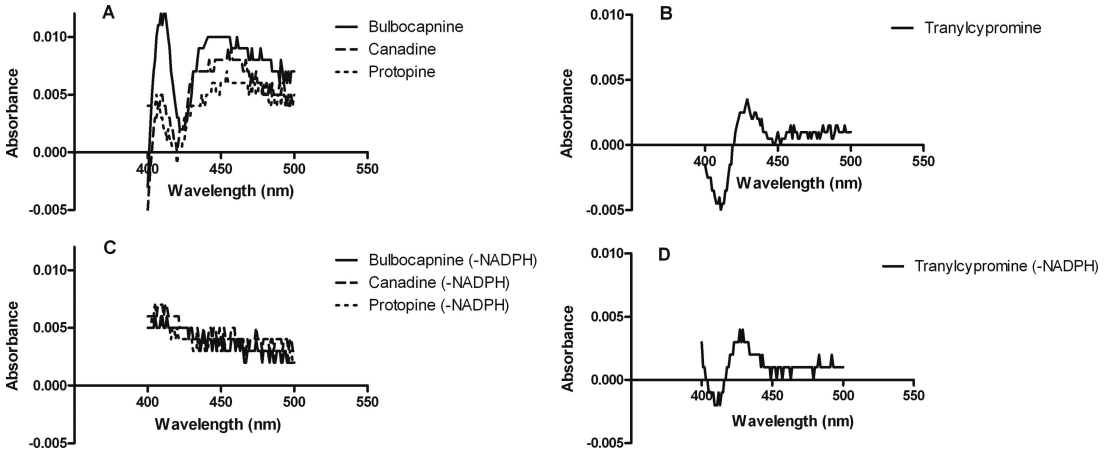


FIG. 3. Representative difference spectra for incubations of human CYP2C19 with isoquinoline alkaloids and the negative control, tranylcypromine, respectively, in the presence (A and B) and absence (C and D) of the NADPH-regenerating system.

approach for identifying novel mechanism-based inactivators of CYP2C19. Three new mechanism-based inactivators of CYP2C19 were introduced. The alkaloids bulbocapnine, canadine, and protopine displayed significant time- and concentration-dependent inactivation of CYP2C19 in the progress curve analysis, allowing for the determination of the two principal kinetic constants k_{inact} and K_I. The analysis also offered mechanistic insights into the inactivation process by revealing saturation of inactivation kinetics, indicative of a two-step inactivation mechanism.

In general, mechanism-based inactivators of various enzymes including P450s have very specific features, which make them recognizable in in vitro tests. Several methods for investigating MBI properties have appeared during the last few decades (Abeles and Maycock, 1976; Silverman, 1995; Kent et al., 2001; Fontana et al., 2005; Polasek and Miners, 2007). However, effective and sophisticated methods for the screening of MBI in P450s are still at an early stage and in vitro-in vivo correlations are limited or impossible without reasonable estimates of k_{inact} and K_I (Ghanbari et al., 2006; Grime et al., 2009; Grimm et al., 2009).

Additional experiments were performed to confirm the MBI nature of inhibition by these alkaloids. The dialysis experiment detected an NADPH-dependent difference in recovery of the enzyme activity after inactivation with bulbocapnine, canadine, and protopine, displaying characteristics similar to those of the positive control isoniazid. It is known that P450 enzymes can oxidize 1,1-disubstituted hydrazines and acyl hydrazines, such as isoniazid, to products that coordinate tightly to the heme iron atom, causing quasi-irreversible inactivation (Hines and Prough, 1980; Muakkassah et al., 1981; Correia and Ortiz de Montellano, 2005), and clinically relevant drug interactions with CYP2C19 substrate drugs have been reported (Nishimura et al., 2003; Kalgutkar et al., 2007). In contrast to irreversible inactivation, quasi-irreversible inactivation in vitro via MIC formation can be reversible after dialysis, because MIC formation does not actually destroy the enzyme. Catalytically active enzymes can also be regenerated under appropriate experimental conditions, i.e., the activity of alkylamine MICs can be disrupted in vitro by oxidation of P450 back to the ferric state using potassium ferricyanide. Under physiological conditions, however, the MIC complexes are known to be so stable that the P450 enzyme(s) involved in the complex formation would be unavailable for drug metabolism, and, hence, the pharmacokinetic impact is indistinguishable from irreversible (suicide) inhibition (Kalgutkar et al., 2007; Polasek and Miners, 2007; Riley et al., 2007; Grimm et al., 2009). For tranylcypromine, the dialysis experiment did not detect any NADPH-dependent difference either in inhibition or recovery of the enzyme activity.

TABLE 3

IC_{50} values for the alkaloids

Alkaloid	IC_{50} Value	
	With Preincubation	Without Preincubation
Bulbocapnine	8.4 ± 1.4 μM (moderately potent)	17.3 ± 6.8 μM (weak)
Canadine	0.29 ± 0.07 μM (potent)	2.2 ± 0.94 μM (moderately potent)
Protopine	1.8 ± 0.33 μM (moderately potent)	2.6 ± 0.48 μM (moderately potent)

Incubation of bulbocapnine, canadine, and protopine caused a NADPH-dependent increase of absorbance at approximately 455 nm (450 nm in the case of bulbocapnine), which is characteristic of the resulting ferrous complex between the heme iron atom and the inhibitory product after the catalytic action of the P450 enzyme, i.e., MIC formation (Kalgutkar et al., 2007). This is presumably due to coordination of a carbene intermediate of methylenedioxy group to heme as is the case with the selective serotonin reuptake inhibitor, paroxetine (Bertelsen et al., 2003; Correia and Ortiz de Montellano, 2005). The absorption spectrum observed with tranylcypromine is characterized by a "type II" binding spectrum with a Soret maximum at 425 to 435 nm and a trough at 390 to 405 nm, and this was independent of the NADPH-regenerating system (Fig. 3). This finding is consistent with previous reports of tranylcypromine interactions being able to elicit a type II spectral response with P450 enzymes (Taavitsainen et al., 2001; Wada et al., 2004). These results indicate that the mechanism of CYP2C19 inhibition by bulbocapnine, canadine, and protopine is quasi-irreversible and mediated via MIC formation.

The IC_{50} determinations were performed according to the guidelines provided by FDA (Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, 2006; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm072101.pdf) concerning TDI and potential MBI, i.e., using a preincubation step

before initiation of the reactions. Increases in potency of CYP2C19 inhibition were observed after this preincubation, causing a ~2-fold reduction in the IC_{50} value of bulbocapnine and protopine and a ~7-fold reduction in the IC_{50} value of canadine. An increase in potency of the P450 inhibitor in the in vitro incubation or dosing period in vivo is a typical feature of TDI (Riley et al., 2007). However, this experiment does not actually provide any additional value for this research, and in the case of reversible inhibition, IC_{50} values could have been determined from the progress curve analysis approach, because this allows investigation of both reversible and irreversible components of the reaction mechanisms (Salminen et al., 2011a).

Several MDP compounds, for example, two marketed drugs, tadalafil and paroxetine, inhibit P450 enzymes CYP3A4 and CYP2D6 via MBI. The thienopyridine antiplatelet drug, ticlopidine, is the first and best-known mechanism-based inactivator of CYP2C19; the inactivation, which occurs by covalent binding of a reactive metabolite of ticlopidine, leads to clinically relevant drug interactions with CYP2C19 substrate drugs (Donahue et al., 1997; Tateishi et al., 1999; Ha-Duong et al., 2001; Venkatakrishnan and Obach, 2007). Bulbocapnine, canadine, and protopine are widely distributed in plants of the family of Papaveraceae (or Fumariaceae, depending on botanical classification), with occurrences in a few species of other families. Hence, they are components present in variable amounts in numerous herbal medicine preparations (Vacek et al., 2010; Gotti, 2011). Protopine is present, e.g., in preparations from the medicinal plant *Fumaria officinalis*, which is included in the European Pharmacopoeia (Vrba et al., 2011). A wide spectrum of biological activities have been reported for all three alkaloids, and their core structures have been used to develop lead molecules for potential treatments against a variety of diseases (Zhang et al., 2007; Jadhav et al., 2010). For example, the protoberberine skeleton has been used to develop antibacterial agents (Jadhav et al., 2010), and bulbocapnine possesses dopamine D_2 receptor antagonist properties (Zhang et al., 2007). These three alkaloids are also present in several traditional Chinese medicine preparations (Salminen et al., 2011b). Thus, products containing these alkaloids have the capacity to inhibit CYP2C19 and interfere with the elimination of the drugs that are substrates of this enzyme.

In conclusion, specific in vitro criteria were used to assess whether three MDP compounds, bulbocapnine, canadine, and protopine, are mechanism-based inactivators of CYP2C19. The applied CYP2C19 progress curve analysis yielded a wealth of information in one run because the quasi-irreversible inactivation pattern by these compounds explained the TDI observed in this analysis, and it provided the principal kinetic constants and mechanistic information of the inactivation process. These herbal medicine alkaloids are novel mechanism-based inactivators for CYP2C19 and may thus interact with drugs that are CYP2C19 substrates. In addition, because a variety of different mechanism-based inactivators are known to be useful in identifying active site amino acid residues involved in substrate binding and catalysis, these alkaloids can serve as good tools for studying structural and functional elements of CYP2C19, especially in the absence of the crystal structure of CYP2C19.

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Authorship Contributions

Participated in research design: Salminen and Raunio.
Conducted experiments: Salminen.
Contributed new reagents or analytic tools: Meyer and Imming.
Performed data analysis: Salminen.
Wrote or contributed to the writing of the manuscript: Salminen, Meyer, Imming, and Raunio.

References

Abeles RH and Maycock AL (1976) Suicide enzyme inactivators. *Acc Chem Res* **9:**313–319.
Bertelsen KM, Venkatakrishnan K, Von Moltke LL, Obach RS, and Greenblatt DJ (2003) Apparent mechanism-based inhibition of human CYP2D6 in vitro by paroxetine: comparison with fluoxetine and quinidine. *Drug Metab Dispos* **31:**289–293.
Casida JE (1970) Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J Agric Food Chem* **18:**753–772.
Correia MA and Ortiz de Montellano PR (2005) Inhibition of cytochrome P450 enzymes, in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed) pp 247–322, Kluwer Academic/Plenum Publishers, New York.
Donahue SR, Flockhart DA, Abernethy DR, and Ko JW (1997) Ticlopidine inhibition of phenytoin metabolism mediated by potent inhibition of CYP2C19. *Clin Pharmacol Ther* **62:**572–577.
Fontana E, Dansette PM, and Poli SM (2005) Cytochrome p450 enzymes mechanism based inhibitors: common sub-structures and reactivity. *Curr Drug Metab* **6:**413–454.
Franklin MR (1971) The enzymic formation of methylenedioxyphenyl derivative exhibiting an isocyanide-like spectrum with reduced cytochrome P-450 in hepatic microsomes. *Xenobiotica* **1:**581–591.
Fukuto JM, Kumagai Y, and Cho AK (1991) Determination of the mechanism of demethylenation of (methylenedioxy)phenyl compounds by cytochrome P450 using deuterium isotope effects. *J Med Chem* **34:**2871–2876.
Ghanbari F, Rowland-Yeo K, Bloomer JC, Clarke SE, Lennard MS, Tucker GT, and Rostami-Hodjegan A (2006) A critical evaluation of the experimental design of studies of mechanism based enzyme inhibition, with implications for in vitro-in vivo extrapolation. *Curr Drug Metab* **7:**315–334.
Gotti R (2011) Capillary electrophoresis of phytochemical substances in herbal drugs and medicinal plants. *J Pharm Biomed Anal* **55:**775–801.
Grime KH, Bird J, Ferguson D, and Riley RJ (2009) Mechanism-based inhibition of cytochrome P450 enzymes: an evaluation of early decision making in vitro approaches and drug-drug interaction prediction methods. *Eur J Pharm Sci* **36:**175–191.
Grimm SW, Einolf HJ, Hall SD, He K, Lim HK, Ling KH, Lu C, Nomeir AA, Seibert E, Skordos KW, et al. (2009) The conduct of in vitro studies to address time-dependent inhibition of drug-metabolizing enzymes: a perspective of the Pharmaceutical Research and Manufacturers of America. *Drug Metab Dispos* **37:**1355–1370.
Guengerich FP (2008) Cytochrome p450 and chemical toxicology. *Chem Res Toxicol* **21:**70–83.
Ha-Duong NT, Dijols S, Macherey AC, Goldstein JA, Dansette PM, and Mansuy D (2001) Ticlopidine as a selective mechanism-based inhibitor of human cytochrome P450 2C19. *Biochemistry* **40:**12112–12122.
Hines RN and Prough RA (1980) The characterization of an inhibitory complex formed with cytochrome P-450 and a metabolite of 1,1-disubstituted hydrazines. *J Pharmacol Exp Ther* **214:**80–86.
Jadhav VB, Nayak SK, Row TN, and Kulkarni MV (2010) Synthesis, structure and DNA cleavage studies of coumarin analogues of tetrahydroisoquinoline and protoberberine alkaloids. *Eur J Med Chem* **45:**3575–3580.
Kalgutkar AS, Obach RS, and Maurer TS (2007) Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interactions and idiosyncratic adverse drug reactions. *Curr Drug Metab* **8:**407–447.
Kent UM, Juschyshyn MI, and Hollenberg PF (2001) Mechanism-based inactivators as probes of cytochrome P450 structure and function. *Curr Drug Metab* **2:**215–243.
Khojasteh SC, Prabhu S, Kenny JR, Halladay JS, and Lu AY (2011) Chemical inhibitors of cytochrome P450 isoforms in human liver microsomes: a re-evaluation of P450 isoform selectivity. *Eur J Drug Metab Pharmacokinet* **36:**1–16.
Muakkassah SF, Bidlack WR, and Yang WC (1981) Mechanism of the inhibitory action of isoniazid on microsomal drug metabolism. *Biochem Pharmacol* **30:**1651–1658.
Murray M (2000) Mechanisms of inhibitory and regulatory effects of methylenedioxyphenyl compounds on cytochrome P450-dependent drug oxidation. *Curr Drug Metab* **1:**67–84.
Nishimura Y, Kurata N, Iwase M, and Yasuhara H (2003) Mechanism-based inactivation of human CYP1A2 and CYP2C19 mediated metabolism by isoniazid. *Drug Metab Rev* **35:**100.
Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J, and Raunio H (2008) Inhibition and induction of human cytochrome P450 enzymes: current status. *Arch Toxicol* **82:**667–715.
Polasek TM and Miners JO (2007) In vitro approaches to investigate mechanism-based inactivation of CYP enzymes. *Expert Opin Drug Metab Toxicol* **3:**321–329.
Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metab Rev* **34:**83–448.
Riley RJ, Grime K, and Weaver R (2007) Time-dependent CYP inhibition. *Expert Opin Drug Metab Toxicol* **3:**51–66.
Ring BJ, Patterson BE, Mitchell MI, Vandenbranden M, Gillespie J, Bedding AW, Jewell H, Payne CD, Forgue ST, Eckstein J, et al. (2005) Effect of tadalafil on cytochrome P450 3A4-mediated clearance: studies in vitro and in vivo. *Clin Pharmacol Ther* **77:**63–75.
Salminen KA, Leppänen J, Venäläinen JI, Pasanen M, Auriola S, Juvonen RO, and Raunio H (2011a) Simple, direct, and informative method for the assessment of CYP2C19 enzyme inactivation kinetics. *Drug Metab Dispos* **39:**412–418.
Salminen KA, Meyer A, Jerabkova L, Korhonen LE, Rahnasto M, Juvonen RO, Imming P, and Raunio H (2011b) Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids. *Phytomedicine* **18:**533–538.
Silverman RB (1995) Mechanism-based enzyme inactivators. *Methods Enzymol* **249:**240–283.
Taavitsainen P, Juvonen R, and Pelkonen O (2001) In vitro inhibition of cytochrome P450 enzymes in human liver microsomes by a potent CYP2A6 inhibitor, *trans*-2-phenylcyclopropylamine (tranylcypromine), and its nonamine analog, cyclopropylbenzene. *Drug Metab Dispos* **29:**217–222.
Tateishi T, Kumai T, Watanabe M, Nakura H, Tanaka M, and Kobayashi S (1999) Ticlopidine

decreases the in vivo activity of CYP2C19 as measured by omeprazole metabolism. *Br J Clin Pharmacol* **47:**454–457.
Vacek J, Walterova D, Vrublova E, and Simanek V (2010) The chemical and biological properties of protopine and allocryptopine. *Heterocycles* **81:**1773–1789.
Venkatakrishnan K and Obach RS (2007) Drug-drug interactions via mechanism-based cytochrome P450 inactivation: points to consider for risk assessment from in vitro data and clinical pharmacologic evaluation. *Curr Drug Metab* **8:**449–462.
Vrba J, Vrublova E, Modriansky M, and Ulrichova J (2011) Protopine and allocryptopine increase mRNA levels of cytochromes P450 1A in human hepatocytes and HepG2 cells independently of AhR. *Toxicol Lett* **203:**135–141.
Wada M, Yokoyama C, Hatae T, Shimonishi M, Nakamura M, Imai Y, Ullrich V, and Tanabe T (2004) Purification and characterization of recombinant human prostacyclin synthase. *J Biochem* **135:**455–463.
Wang JF, Zhang CC, Chou KC, and Wei DQ (2009) Structure of cytochrome p450s and personalized drug. *Curr Med Chem* **16:**232–244.
Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4:**825–833.
Zhang A, Zhang Y, Branfman AR, Baldessarini RJ, and Neumeyer JL (2007) Advances in development of dopaminergic aporphinoids. *J Med Chem* **50:**171–181.

Address correspondence to: Dr. Kaisa A. Salminen, School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland. E-mail: kaisa.salminen@uef.fi

IV

Time-dependent inhibition of CYP2C19 by isoquinoline alkaloids: in vitro and in silico analysis

Salminen KA, Rahnasto-Rilla M, Väänänen R, Imming P, Meyer A, Horling A, Poso A, Laitinen T, Raunio H and Lahtela-Kakkonen M

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Time-Dependent Inhibition of CYP2C19 by Isoquinoline Alkaloids: In Vitro and In Silico Analysis

Kaisa A. Salminen, Minna Rahnasto-Rilla, Raija Väänänen, Peter Imming, Achim Meyer, Aline Horling, Antti Poso, Tuomo Laitinen, Hannu Raunio, and Maija Lahtela-Kakkonen

School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, Kuopio, Finland (K.A.S., M.R.-R., R.V., A.P., T.L., H.R., M.L.-K.); and Institut für Pharmazie, Martin-Luther-Universität Halle-Wittenberg, Halle, Germany (P.I., A.M., A.H.)

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ABSTRACT

The cytochrome P450 2C19 (CYP2C19) enzyme plays an important role in the metabolism of many commonly used drugs. Relatively little is known about CYP2C19 inhibitors, including compounds of natural origin, which could inhibit CYP2C19, potentially causing clinically relevant metabolism-based drug interactions. We evaluated a series (N = 49) of structurally related plant isoquinoline alkaloids for their abilities to interact with CYP2C19 enzyme using in vitro and in silico methods. We examined several common active alkaloids found in herbal products such as apomorphine, berberine, noscapine, and papaverine, as well as the previously identified mechanism-based inactivators bulbocapnine, canadine, and protopine. The IC_{50} values of the alkaloids ranged from 0.11 to 210 μM, and 42 of the alkaloids were confirmed to be time-dependent inhibitors of CYP2C19. Molecular docking and three-dimensional quantitative structure-activity relationship analysis revealed key interactions of the potent inhibitors with the enzyme active site. We constructed a comparative molecular field analysis model that was able to predict the inhibitory potency of a series of independent test molecules. This study revealed that many of these isoquinoline alkaloids do have the potential to cause clinically relevant drug interactions. These results highlight the need for studying more profoundly the potential interactions between drugs and herbal products. When further refined, in silico methods can be useful in the high-throughput prediction of P450 inhibitory potential of pharmaceutical compounds.

Introduction

Plant alkaloids are a highly diverse group of natural compounds containing a ring structure and one or more basic nitrogens. Alkaloids often have marked pharmacologic activity, and plants containing alkaloids have traditionally been used worldwide as a source of medicinal products. Some alkaloids have been successfully developed into widely used pharmaceuticals (e.g., morphine, codeine, apomorphine, papaverine, emetine, noscapine) (Gotti, 2011; Newman and Cragg, 2012; Hagel and Facchini, 2013).

Natural herbal products, including traditional Chinese medicine (TCM) preparations, are becoming increasingly popular even in Western countries (Gotti, 2011; Salminen et al., 2011b; Pelkonen et al., 2014). It is often claimed that herbal medicines are harmless because of their natural origin and long-term use. Nevertheless, it is evident that these products can cause a variety of adverse effects (Izzo and Ernst, 2009; Jordan et al., 2010; Singh et al., 2012) and may elicit numerous clinically significant drug interactions (Tsai et al., 2012).

Human liver cytochrome P450 (P450) enzymes metabolize a large variety of compounds, including many synthetic and natural medicines (Wienkers and Heath, 2005; Guengerich, 2008). Although the CYP2C19 enzyme catalyzes the metabolism of fewer drug substrates than other enzymes in its family (e.g., CYP3A4 and CYP2D6 enzymes), it plays an important role in the metabolism of some proton pump inhibitors, antidepressants, antipsychotics, antiepileptics, and antiplatelet drugs (Rendic, 2002; Pelkonen et al., 2008; Wang et al., 2009). In fact, the popular platelet antagonist clopidogrel is a prodrug activated by CYP2C19 (Scott et al., 2013).

Inhibition of P450 enzymes is a major mechanism for pharmacokinetic drug interactions (Wienkers and Heath, 2005; Boobis et al., 2009). In general, P450 enzymes are inhibited either as reversible inhibition or via the more profound and prolonged irreversible or quasi-irreversible inactivation. Irreversible or quasi-irreversible mechanism-based (i.e., P450 catalysis-dependent) inactivation is of greater practical concern because of its serious risk of causing clinically significant drug interactions. Time-dependent inhibition (TDI) is one of the major features that distinguish between reversible inhibition and irreversible or quasi-irreversible mechanism-based inactivation.

Several components in herbal medicines have been shown to inhibit various members of the P450 enzyme family (Izzo and Ernst, 2009; Posadzki et al., 2013). The methylenedioxyphenyl (MDP) moiety is present in many plant compounds, including alkaloids; these secondary metabolites are intended to deter the foraging by predatory insects and herbivores (Murray, 2012). There are reports that compounds containing the MDP moiety can inactivate P450 enzymes as a result of the formation of a metabolite intermediate complex (MIC) with the enzyme, leading to quasi-irreversible enzyme inactivation (Casida, 1970; Franklin, 1971; Nakajima et al., 1999; Correia and Ortiz de Montellano, 2005). With the increasing popularity of herbal medicinal

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ABBREVIATIONS: 3D-QSAR, three-dimensional quantitative structure-activity relationship; CoMFA, comparative molecular field analysis; MDP, methylenedioxyphenyl; MIC, metabolic intermediate complex; P450, cytochrome P450; PLS, partial least square; TCM, traditional Chinese medicine; TDI, time-dependent inhibition.

products, human exposure to plant compounds with the MDP moiety may become significant.

Drug interaction potential is today routinely screened during the drug development process. The evaluation of TDI properties in drug candidates is currently recommended by the European Medicines Agency (EMA, Guideline on the Investigation of Drug Interactions, 2012, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf) and the U.S. Food and Drug Administration (FDA, Guidance for Industry: Drug Interaction Studies – Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, 2012, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf). Furthermore, the evaluation of potential drug interactions is recommended also for new herbal preparations.

We have previously evaluated the inhibitory characteristics of several natural and chemically derived isoquinoline alkaloids toward all the principal human drug-metabolizing P450 enzymes. Many alkaloids proved to be potent inhibitors of the CYP2C19, CYP2D6, and CYP3A4 forms, and three (i.e., protopine, bulbocapnine, and canadine) were identified as mechanism-based inactivators of CYP2C19 (Salminen et al., 2011b,c). Although several mechanism-based inactivators have been identified for P450s, few attempts have been made to evaluate these compounds by exploiting molecular modeling (in silico) approaches. Several quantitative structure-activity relationship (QSAR) models have been published for CYP2C19 ligands (Suzuki et al., 2002, 2004; Lewis, 2004; Lewis et al., 2006; Gleeson et al., 2007; Foti et al., 2012), but thus far, none has specifically addressed mechanism-based inactivators.

In this study, we evaluated putative interactions of structurally related isoquinoline alkaloids with the CYP2C19 enzyme using in vitro and in silico approaches. Special attention was paid to the inhibition of CYP2C19 by alkaloids containing the MDP moiety. Molecular modeling, including docking and a three-dimensional quantitative structure-activity relationship (3D-QSAR) analysis, was conducted to devise a model for prediction of inhibitory activity of alkaloids.

Materials and Methods

Chemicals

The isoquinoline alkaloids **1**–**8**, **10**–**17**, and **20**–**39** have been described in our previous studies (Meyer and Imming, 2011; Salminen et al., 2011b); alkaloids **40** and **42** are synthetic, and **9** and **41** are semisynthetic derivatives of **23** (tetrahydropalmatine) (unpublished data). Alkaloids **18**–**19** and **43**–**49** were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Substrate dibenzylfluorescein (DBF) (purity > 99%) and cDNA-expressed human wild-type CYP2C19 (Supersomes) (with cytochrome b5) were purchased from BD Biosciences Discovery Labware (Bedford, MA). Isoniazid and trans-2-phenylcyclopropylamine hydrochloride (tranylcypromine) were purchased from Sigma-Aldrich and were of the highest purity available.

Inhibition of CYP2C19 Activity

The IC_{50} values for the alkaloids were determined essentially as described previously (Salminen et al., 2011a,b). In short, the assay procedure was used in two steps. First, four to five different concentrations (mainly 1:10 ratio) of each alkaloid at a concentration range of 0.05–1000 μM (water-soluble compounds up to 6.67 mM) were used for prescreening. After that, a more precise concentration range (seven different concentrations, mainly 1:3 ratio) was used in the kinetic assay procedure without substrate premeasurement. In the standard preincubation assay, a mixture containing the inhibitor, enzyme (1.5 pmol), and an NADPH-regenerating system was first preincubated for 30 minutes at 37°C, after which the reactions were initiated by the addition of the substrate at a concentration corresponding to its apparent K_m (1 μM DBF). The IC_{50} values were compared with those obtained in similar experimental conditions without preincubation (Salminen et al., 2011b). All IC_{50} values are reported as means from duplicate determinations. If poor solubility of a compound prevented determination of exact IC_{50} shift, the fluorometric CYP2C19 progress curve analysis was applied (Salminen et al., 2011c). The progress curve analysis assay was performed in duplicate for compounds **2**, **14**, **17**, **22**, **27**, **31**, **33**, **34**, **37**, **38**, and **39.** The curves were visually inspected to determine whether a compound exhibited time- and concentration-dependent inhibition. The known time-dependent CYP2C19 inhibitor isoniazid and the known reversible inhibitor tranylcypromine were used as positive controls.

Molecular Modeling.

Structure Preparation. The crystal structure of human CYP2C19 with the inhibitor 2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl)methanon (PDB: 4GQS) (Reynald et al., 2012) was used for molecular docking studies. The protein structure was processed with the Protein Preparation Wizard of Schrödinger Maestro version 9.6 (Schrödinger, 2013) using standard settings (add hydrogens, assign bond orders, create zero-order bonds to metals and disulfide bonds, and delete waters beyond 5 Å from heteroatoms). Hydrogen bonds were assigned, and the prepared structure was minimized using OPLS_2005 force field and restrained minimization (heavy atom converging RMSD 0.30 Å). The missing side chains were added to the crystal structure using Prime version 3.0 (Schrödinger, 2012a).

The crystal structures for the following alkaloids were retrieved from the Cambridge Structural Database (CSD) using ConQuest program (Allen, 2002; Bruno et al., 2002): **4** (reference code GEMGAG), **12** (SONCUT), **17** (SOKCOK), **18** (BICUCL01), **23** (WASSIS01), **33** (CORALY), **36** (MEGVOK), **44** (FINPUN), **45** (VIGFEW), and **47** (CADBUE). The rest of the compounds were constructed using SYBYL-X 2.0 (Tripos International, 2011). All compounds were preprocessed with LigPrep v. 2.5 (Schrödinger, 2011); the molecules were desalted, tautomers were determined at pH 7 $\pm$ 1 using Epik, and all possible enantiomers (or a maximum of 32) were created if chirality was not present in the original structures.

Molecular docking. Glide version 5.8 (Friesner et al., 2004; Halgren et al., 2004; Friesner et al., 2006; Schrödinger, 2012b) was used to dock alkaloids in the active site of CYP2C19. The Glide grid-file was constructed to represent the shape and properties of the crystal structure of CYP2C19 (PDB: 4GQS) to provide more accurate scoring of the ligands. The center of the grid was defined based on the cocrystalized ligand (2-methyl-1-benzofuran-3-yl)-(4-hydroxy-3,5-dimethylphenyl) methanon) of CYP2C19 to determine the position of the active site. A sufficiently large grid 20 Å long was defined to ensure that there is enough space for large ligands. The metal coordination constraint of heme iron and the positional constraints that define the spherical region occupied by inhibitors were applied. These constraints were used to ensure that the ligands were orientated close to the heme. The flexible docking studies were carried out for all alkaloids. The docking results were visually inspected, and the highest scoring docking poses in which MDP moiety is orientated close to the heme iron were chosen for 3D-QSAR evaluation using the Comparative Molecular Field Analysis (CoMFA) method with Sybyl-X 2.0 (Cramer et al., 1988; Tripos International, 2011).

CoMFA models. Molecular docking was used to align 49 alkaloids in the conformation shown in the crystal structure of 4GQS. The alkaloids were divided to a training set (**1–8, 10–17,19–23, 25–27, 29, 31–32, 34–41, 43–44**) (n = 37) and a test set (**9, 18, 24, 28, 30, 33, 42, 45–49**) (n = 12). The test set contained alkaloids with diverse activities (IC_{50} 1.4–150 μM) and structures. The steric and electrostatic fields were calculated for the alkaloids. Multiple partial least square (PLS) models were built and validated using the cross-validation and progressive scrambling methods to obtain the optimum number of PLS components for the final analysis. The leave-one-out (LOO) and cross-validation models with different numbers of components (one to five components) and with different group sizes (two and five groups) were carried out with column filtering set to 2.0 (Supplementary Material Tables S1–S3). The final analysis was done with three components.

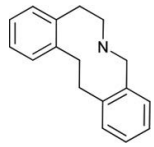
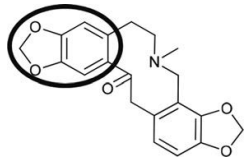
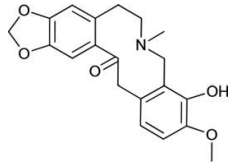
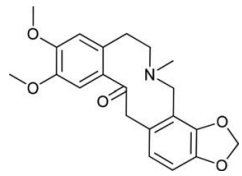
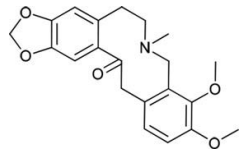
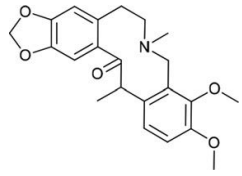
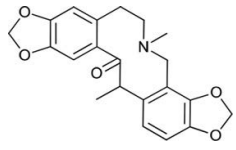
Results

Inhibition of CYP2C19 Activity. The IC_{50} values determined using a 30-minute preincubation and TDI characteristics (i.e., a shift-down in IC_{50} with 30-minute preincubation or TDI in the progress curve analysis) are presented in Table 1. For most compounds (i.e., 39 of 49, 79.6%), the IC_{50} value decreased $\geq$1.5-fold with the preincubation; thus, these compounds were classified as time-dependent inhibitors. Time- and concentration-dependent inhibition was confirmed by the

TABLE 1

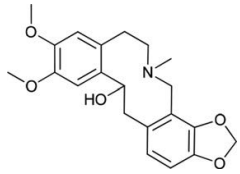
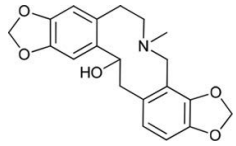
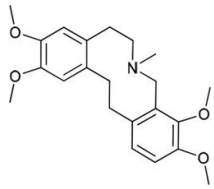
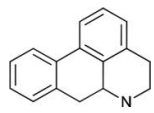
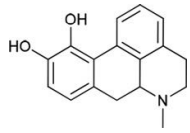
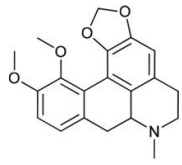
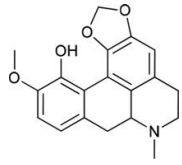
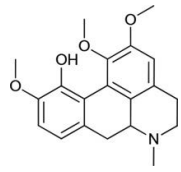
IC_{50} values determined with 30-minute preincubation and inhibition characteristics for the alkaloids organized according to the isoquinoline alkaloid subgroups.

The methylenedioxyphenyl (MDP) moiety is shown in **1** (protopine) inside the circle.

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
	Protopines			
1	Protopine		1.8 (1.1–2.8)	1.5
2	Hunnemannine		79 (63–101)	N.D.
3	Cryptopine		0.66 (0.53–0.82)	4.9
4	Allocryptopine		76 (37–157)	1.9
5	Corycavidine (13S)		15 (6.5–36)	6.3
6	Corycavamine (13R)		0.19 (0.13–0.27)	6.4

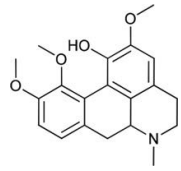
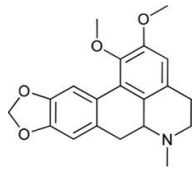
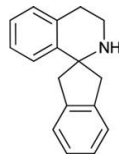
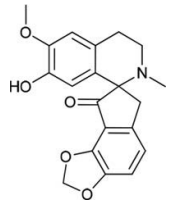
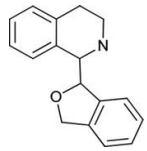
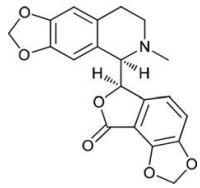
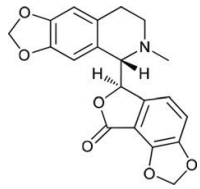
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
7	Dihydrocryptopine[d](racemic)		0.35 (0.23–0.53)	3.5
8	Dihydroprotopine[d](racemic)		0.11 (0.078–0.16)	2.7
9	2,3-Dimethoxy-7-methyl-9, 10-dimethoxy-5,6,7,8,13, 14-hexahydrodibenzo [d,h,] azecine		23 (10–53)	>4.3
	Aporphines			
10	Apomorphine (R)		7.8 (4.7–13)	2.3
11	Bulbocapninemethylether[d] (S)		61 (48–77)	1.5
12	Bulbocapnine (S)		8.4 (5.7–12)	2.1
13	Isocorydine (S)		33 (20–54)	1.9

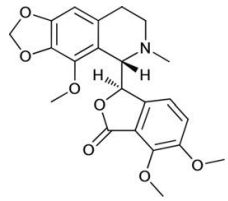
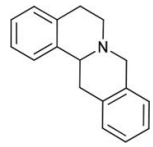
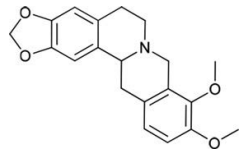
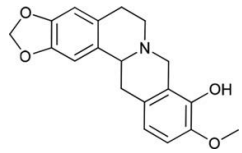
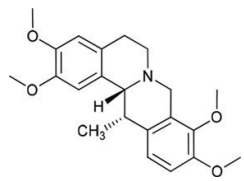
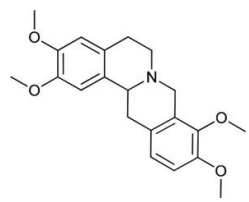
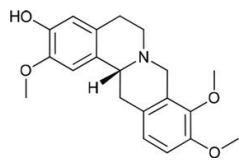
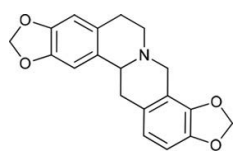
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
14	Corydine (S)		91 (51–162)	TDI
15	Nantenine (S)		3.5 (2.4–5.2)	N.D.
	Spirobenzylisoquinolines			
16	Parfumine (S)		6.3 (3.7–11)	2.4
	Phthalideisoquinolines			
17	Capnoidine (1R, 9R)		0.47 (0.37–0.60)	TDI
18	Bicuculline (1S, 9R)		150 (91–246)	9.5

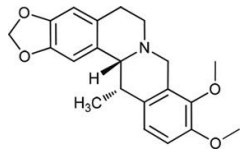
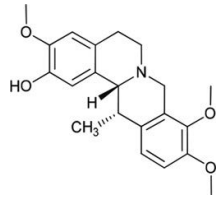
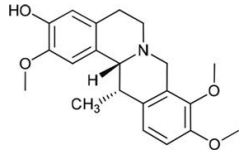
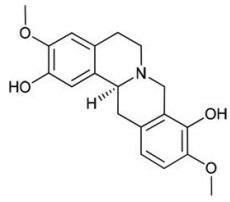
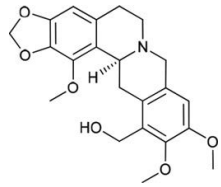
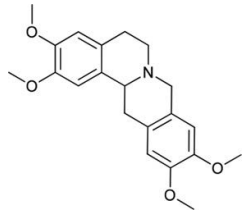
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
19	Noscapine (3S, 5R)		0.20 (0.13–0.30)	5.0
	Protoberberines			
20	Canadine[d](racemic)		0.29 (0.20–0.47)	7.6
21	Nandinine (racemic)		0.37 (0.30–0.47)	10.1
22	Corydaline (13S, 13aR)		3.1 (2.0–4.8)	TDI
23	Tetrahydropalmatine (13aR)		7.0 (6.0–8.1)	3.0
24	Corypalmine (13aS)		8.9 (0.1–18.5)	N.D.
25	Stylopine[d](racemic)		0.13 (0.083–0.19)	4.5

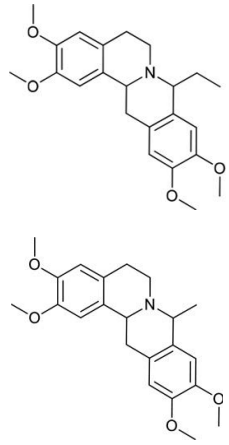
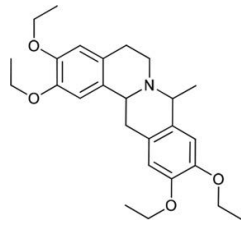
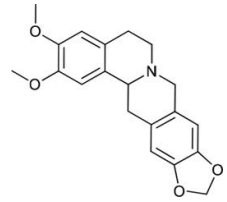
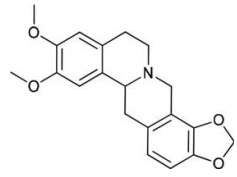
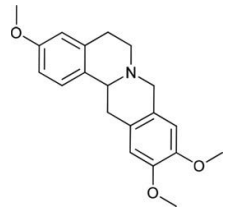
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
26	Thalictricavine (13S, 13aR)		2.4 (1.7–3.3)	4.5
27	Isocorybulbine (13S, 13aR)		210 (141–324)	N.D.
28	Corybulbine (13S, 13aR)		5.4 (3.6–8.1)	3.4
29	Scoulerine (13aS)		22 (13–38)	3.3
30	Mecambridine (13aS)		21 (15–31)	4.7
31	2,3,10,11-Tetramethoxyberbine[e](racemic)		190 (141–258)	N.D.

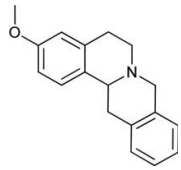
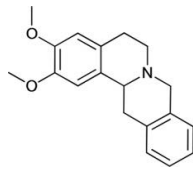
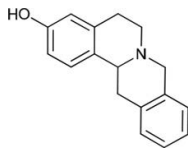
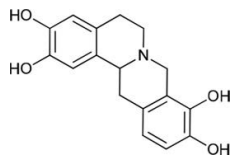
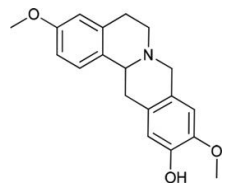
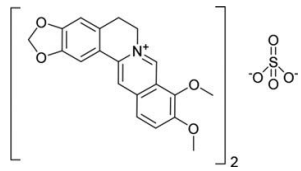
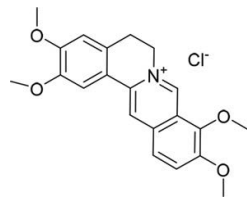
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
32	8-Ethyl-2,3,10,11-tetramethoxyberbine[e] (racemic)		31 (23–43)	4.8
33	8-Methyl-2,3,10,11-tetramethoxyberbine[e] (racemic)		130 (97–176)	N.D.
34	8-Methyl-2,3,10,11-tetraethoxyberbine[e] (racemic)		59 (39–87)	N.D.
35	2,3-Dimethoxy-10,11-methylenedioxyberbine[e] (racemic)		2.3 (1.7–3.1)	3.8
36	2,3-Dimethoxy-9,10-methylenedioxyberbine[e] (racemic)		5.0 (4.2–6.0)	4.5
37	3,10,11-Trimethoxyberbine[e] (racemic)		25 (11–58)	TDI

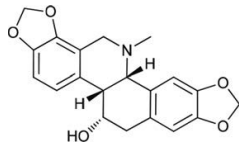
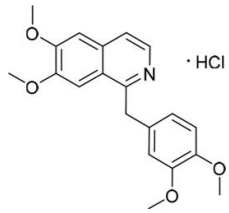
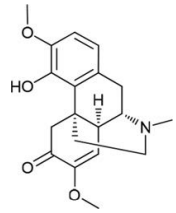
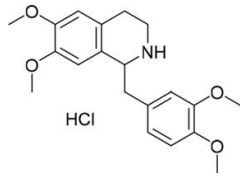
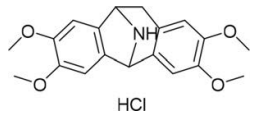
(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
38	3-Methoxyberbine[e](racemic)		2.4 (1.2–5.0)	TDI
39	2,3-Dimethoxyberbine[e](racemic)		40 (28–56)	TDI
40	3-Hydroxyberbine[e](racemic)		14 (10–18)	>7
41	2,3,9,10-Tetrahydroxyberbine(racemic)		19 (12–30)	1.5
42	3,10-Dimethoxy-11-hydroxyberbine[e](racemic)		11 (3.6–35)	>8.9
				
43	Berberine hemisulfate		150 (42–298)	2.3
44	Palmatine		120 (84–185)	2.9

(*continued*)

TABLE 1—*Continued*

No.	Compound (Stereochemistry)	Structure	Shifted IC_{50}[a] (95% CI[b]) (μM)	IC_{50}-fold Shift or TDI[c]
	Others			
45	Chelidonine (5bR, 6S, 12bS)		1.4 (1.1–1.7)	8.4
46	Papaverine hydrochloride		2.2 (1.6–3.2)	1.6
47	Sinomenine (9aR, 13aS, 14aS)		55 (37–83)	11.5
48	Norlaudanosine hydrochloride (racemic)		13 (11–15)	3.3
49	Pavine hydrochloride (racemic)		82 (70–97)	3.9

N.D., not determined.
[a]Determined with 30-minute preincubation.
[b]Confidence intervals.
[c]Time-dependent inhibition by progress curve analysis.
[d]Semisynthetic.
[e]Synthetic.

progress curve analyses for alkaloids **14**, **17**, **22**, **37**, **38**, and **39**. In addition, K_I and k_{inact} values were calculated for **17** (2.8 ± 0.69 μM and 0.144 ± 0.009 minute^{-1}), **22** (68 ± 23 μM and 0.286 ± 0.057 minute^{-1}), **38** (77 ± 18 μM and 0.129 ± 0.017 minute^{-1}), and **39** (358 ± 89 μM and 0.094 ± 0.010 minute^{-1}). Insufficient solubility of **2**, **27**, **31**, **33**, and **34** made it impossible to obtain precise results for these compounds in the IC_{50} determination without preincubation (and thus in the IC_{50} shift assay) and in the progress curve analysis. Ultimately, 42 of 49 (86%) of the alkaloids were confirmed to demonstrate TDI of CYP2C19 activity. The known time-dependent inhibitor isoniazid and the known reversible inhibitor tranylcypromine were used as control compounds. As expected, on preincubation, the IC_{50} of isoniazid was reduced up to 10-fold, and that of tranylcypromine was unaffected or increased up to 2-fold.

The most potent CYP2C19 inhibition was exhibited by the protopine, protoberberine, and phthalideisoquinoline alkaloids. In contrast, none of the aporphine alkaloids displayed evidence of potent inhibition (Table 1). The most potent inhibitor was dihydroprotopine **8**, which had an IC_{50} value of 0.11 μM. Other potent inhibitors ($IC_{50} < 1$ μM) were compounds **3** (cryptopine), **6** (corycavamine), **7** (dihydrocryptopine), **17** (capnoidine), **19** (noscapine), **20** (canadine), **21** (nandinine), and **25** (stylopine).

The moderately potent inhibitors (IC_{50} 1–10 μM) were compounds **1** (protopine), **10** (apomorphine), **12** (bulbocapnine), **15** (nantenine), **16** (parfumine), **22** (corydaline), **23** (tetrahydropalmatine), **24** (corypalmine), **26** (thalictricavine), **28** (corybulbine), **35** (2,3-dimethoxy-10,11-methylenedioxyberbine), **36** (2,3-dimethoxy-9,10-methylenedioxyberbine), **38** (3-methoxyberbine), **45** (chelidonine), and **46** (papaverine).

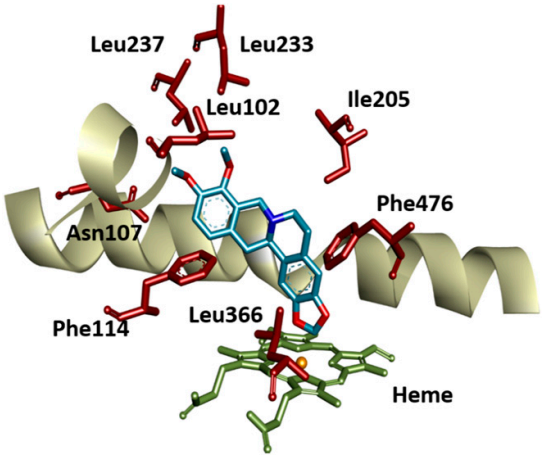


Fig. 1. Compound **20** (canadine) is surrounded by hydrophobic residues Leu102, Phe114, Ile205, Leu233, Leu237, and Phe476 in the CYP2C19 active site. A polar interaction is formed with Asn107.

All the potent inhibitors ($IC_{50} < 1$ μM) possess the MDP moiety, whereas 50% of the moderately potent inhibitors (IC_{50} 1–10 μM) and 28% of weakly inhibitory alkaloids ($IC_{50} > 10$ μM) contained the MDP. Those alkaloids with two MDP moieties ($n = 7$) were among the most potent ($IC_{50} < 2$ μM) inhibitors, with the exception of bicuculline **18** (IC_{50} 150 μM).

Molecular Modeling. Compound **20** (IC_{50} 0.29 μM) was used as the template for docking analysis because of its potency and rigid molecular structure. The docking studies revealed that the MDP in **20** favorable orientated toward the heme iron (Fig. 1). These compounds lie in the hydrophobic pocket formed by residues Leu102, Leu233, Leu237 and Ile205 at the entrance of the active site. In addition, a polar interaction was observed between **20** and a hydrophilic residue (Asn107) in close proximity to the active site. For most of the other alkaloids, the hypothetical orientations of docking poses were similar to that of **20**. The hydrophilic residue Asp293, which is situated close to the active site of the enzyme, can also form a polar interaction with the studied alkaloids.

The CoMFA analysis was carried out with the PLS algorithm. The optimal number of components in the final model was three, which was obtained using the cross-validation technique. The model displayed good statistical values: $r^2 = 0.94$, S.E. estimate of 0.25. The progressive scrambling analysis indicated three components as being optimal based on the minimum value for cSDEP with maximum q^2 value (Supplementary Table S3). The contributions of steric and electrostatic fields of the CoMFA model are 47% and 58%, respectively. The contour maps of the CoMFA models (Fig. 2) represent electronegative (red contour) and electropositive (blue contour) favorable regions, as well as sterically favorable (green contours) and sterically unfavorable regions (yellow contours) for CYP2C19 inhibition. The sterically favorable and unfavorable regions indicated the optimal volume and shape of the inhibitors. The steric fields suggested that the inhibition potency increased if a hydrophobic group could occupy these regions. The CoMFA contour maps, together with canadine **20** and corycavamine **6**, are shown in Fig. 2, A and B, respectively. Overall, the steric and electrostatic contributions of the CoMFA are consistent with the shape and properties of the CYP2C19 active site. Compound **6**, representing a potent MDP derivative, was observed to coordinate toward heme (Fig. 2B). The molecule contains two MDP moieties; one of these points to the contour of the sterically favorable region in the hydrophobic pocket near to residues Leu102, Leu205, Leu233, and Leu237.

The ultimate test for the robustness of a QSAR model is the ability to predict the biologic activity of compounds that have not been included in the training set. An external test set of 12 compounds, including alkaloids covering all inhibition potency categories and various structural scaffolds, was used to evaluate the predictive ability of the model. The correlation between predicted and experimental inhibitory activities ($r^2 = 0.8$) is shown in Fig. 3.

The predictions were within 0.5 log units of the experimental values, with the exception of two compounds (**17** and **18**); however, the predicted pIC_{50} values were in agreement with the experimental data (Supplementary Table S4). The CoMFA contour maps with compounds

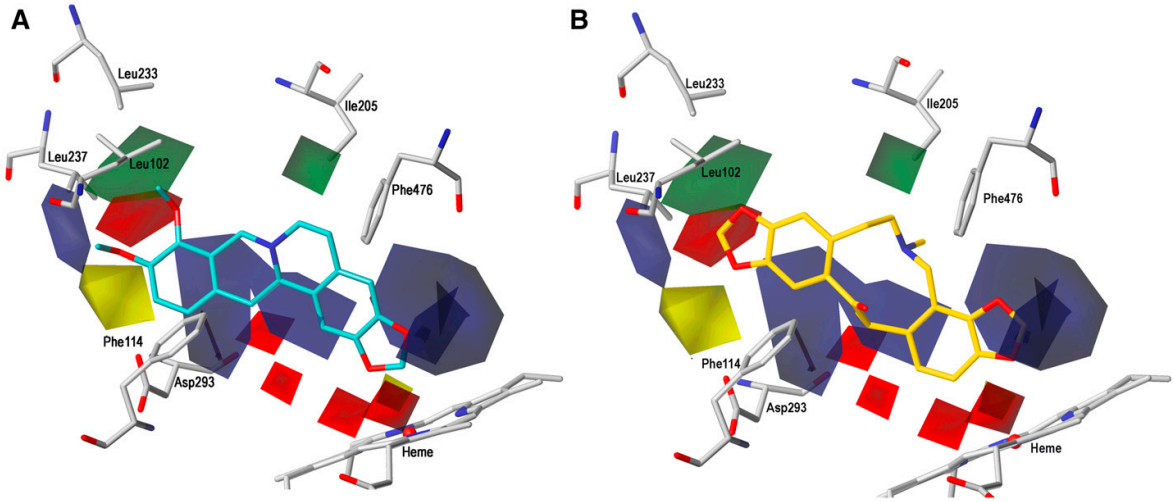


Fig. 2. The contour maps with compounds **20** (A) and **6** (B). The electronegative (red contour) and electropositive (blue contour) favorable regions for inhibition are presented. The steric contours indicate areas where bulky groups increase (green contours) or decrease (yellow contours) inhibitory activity, respectively.

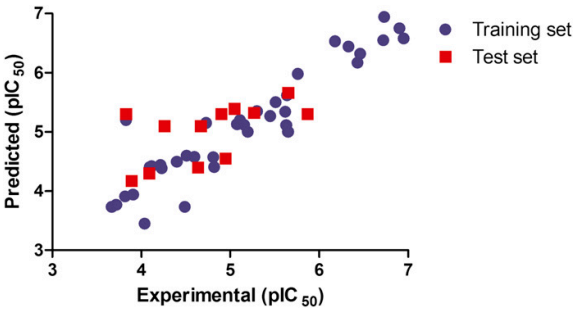


Fig. 3. Graphical interpretation of predicted versus observed pIC_{50} of the training set (blue ball) and test set (red square) alkaloids from CoMFA analysis.

17 and **18** (Fig. 4) showed that these compounds could not be orientated properly toward the heme in the active site of CYP2C19, with the MPD moiety in **18** pointing to a sterically unfavored region (yellow contour).

Discussion

The present results provide information about (1) the potential of a series of plant alkaloids to interfere with metabolism of drugs metabolized by CYP2C19, and (2) the interaction of these ligands with the active site of the CYP2C19 enzyme.

Several (9/49) of the studied alkaloids were found to be potent time-dependent inhibitors of CYP2C19 ($IC_{50} < 1$ μM). In this study, the presence of the MDP moiety increased the inhibitory potency. The IC_{50} shift assay is a straightforward and widely used test to detect TDI while the progress curve analysis method has been more recently applied for P450s (Berry and Zhao, 2008; Salminen et al., 2011a; Stresser et al., 2014). Using these approaches, most of the studied alkaloids (42/49) demonstrated time- and concentration-dependent inhibition. The MDP moiety of most of the alkaloids was coordinated toward the heme. Three TCM compounds with MDP, protopine (**1**), bulbocapnine (**12**), and canadine (**20**), were shown to cause quasi-irreversible inactivation of CYP2C19 mediated via MIC formation in our previous study (Salminen et al., 2011c). These compounds contain a MDP moiety orientated toward the heme in the CYP2C19 active site in the present molecular docking analysis.

We made a detailed search from the commercial TCM Database: Traditional Chinese Medicines: Molecular Structures, Natural Sources, and Applications (NiceData Software, 2005). The database includes 10,458 individual compounds isolated from TCM plants and 4636 TCM medicinal plants. The database also describes their therapeutic utilities. 26 of the 49 investigated alkaloids (**1**, **3**, **4**, **6**, **11**, **12**, **13**, **14**, **15**, **17**, **20**, **21**, **22**, **23**, **24**, **25**, **28**, **29**, **30**, **31**, **36**, **43**, **44**, **45**, **46**, and **47**) were found in the database. These 26 alkaloids were related to numerous TCM plants and medicinal plants. Fifteen individual alkaloids (**1**, **3**, **4**, **12**, **13**, **14**, **17**, **20**, **30**, **31**, **43**, **44**, **45**, **46**, **47**) were reported to have one or more pharmacologic or therapeutic effects. At least 28 of the studied alkaloids (these 26 plus apomorphine and noscapine) are likely to be ingested by humans.

For example, noscapine (**19**), a phthalideisoquinoline alkaloid isolated from opium and widely used as an antitussive drug, has been associated with serious drug interactions when coadministered with warfarin (Ohlsson et al., 2008; Scordo et al., 2008; Fang et al., 2010). It has been demonstrated to inhibit CYP2C9 and CYP3A4 in vitro in a time-dependent fashion (Fang et al., 2010). In an in vivo study, noscapine caused marked inhibition of CYP2C9 and CYP2C19 activity (Rosenborg et al., 2010). The potent inhibition (IC_{50} 0.2 μM), the presence of TDI, and noscapine's orientation in the active site of CYP2C19 observed in the present study are consistent with the proposal that this drug possesses the ability to affect the CYP2C19 pathway in vivo.

Two moderately potent TCM alkaloids, chelidonine (**45**, IC_{50} 1.4 μM), used as a cancer chemotherapeutic drug (Panzer et al., 2001), and papaverine (**46**, IC_{50} of 2.2 μM), used for treatment of vasospasm (Komotar et al., 2007), may also have the capability to affect the CYP2C19-mediated metabolism of other drugs. Based on the present data, two TCM compounds, the noninhibitor berberine

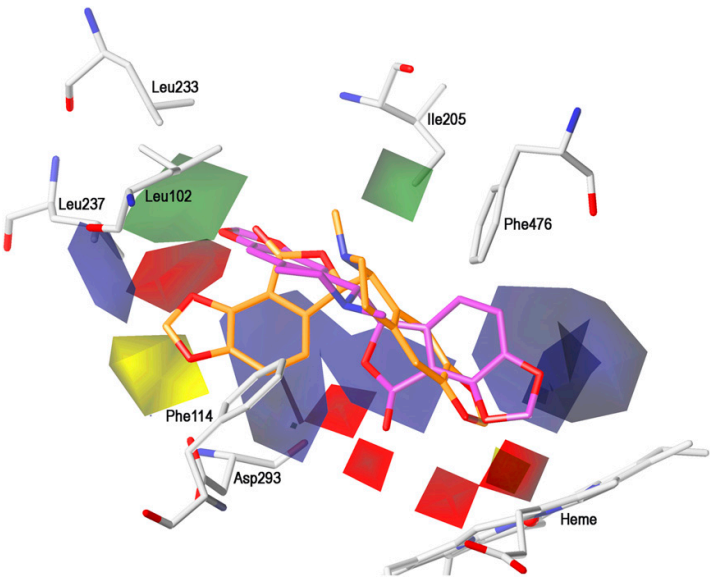


Fig. 4. The contour maps with compounds **17** (purple) and **18** (orange) in the active site of CYP2C19. The contours represent the electronegative (red contour) and electropositive (blue contour) regions and sterically favorable (green contours) and sterically unfavorable regions (yellow contours).

(**43**, IC_{50} 150 μM) and the weak CYP2C19 inhibitor sinomenine (**47**, IC_{50} 55 μM), are unlikely to affect the metabolism of drugs that are substrates of CYP2C19. Berberine (**43**) has been extensively studied and is a widely used constituent in herbal products (Vuddanda et al., 2010; Guo et al., 2012). The observed low CYP2C19 inhibitory potency (IC_{50} 150 μM) is consistent with previous in vitro (Han et al., 2011) and in vivo studies in humans (Guo et al., 2012). Sinomenine (**47**), extracted from the Chinese medical plant *Sinomenium acutum*, is used for the treatment of a variety of disorders such as rheumatism and cardiac arrhythmia. Previously, it has been reported that sinomenine inhibited the activity of CYP2C19 in human microsomes, and in an in vivo study at normal clinical doses, sinomenine enhanced the elimination of mephenytoin, a marker of CYP2C19 activity (Yao et al., 2007).

Molecular docking and predictive CoMFA models were generated to pinpoint steric and electrostatic interactions between the alkaloids and the CYP2C19 active site. Molecular docking is a common approach used to predict the bioactive conformation to generate CoMFA models (Rahnasto et al., 2011). Different types of constraints are often used to improve the docking into the P450 enzymes (Rahnasto et al., 2011). Here, the alkaloids were docked into the X-ray structure of CYP2C19, as it has been observed that a 3D-QSAR model based on X-ray structure can achieve a much better performance and improved statistical significance (Urniaż and Jóźwiak, 2013). The CoMFA steric and electrostatic field maps of the alkaloids were consistent with the crystal structure of CYP2C19 active site.

The progressive scrambling method was applied to address possible overly optimistic cross-validation (Clark and Fox, 2004). A QSAR model is considered to be predictive if the following conditions are satisfied: $r^2 > 0.6$, $q^2 > 0.5$ (Golbraikh et al., 2003). According to these criteria, the present CoMFA model ($r^2 = 0.9$ and $q^2 = 0.6$) has a high goodness-of-fit, robustness and predictive ability. The CoMFA model training set consisted of divergent alkaloids possessing a wide range of inhibition potency. A clear correlation was found between the structures and inhibition potency; however, some subgroups of alkaloids had only a few representatives. The model did not predict satisfactorily the inhibition potency of two alkaloids, **17** and **18**, which together with compound **19** belong to a small and divergent subgroup of phthalideisoquinoline alkaloids with extensive variations in their inhibitory potencies.

In conclusion, we identified several plant alkaloids containing the MDP moiety to be potent time-dependent inhibitors of the CYP2C19 enzyme. Molecular docking and 3D-QSAR analysis revealed key interactions between the alkaloids and the CYP2C19 active site. A CoMFA model was constructed that was capable of predicting inhibition potency. These results suggest that clinically significant drug interactions could well occur when some of these alkaloids are used concomitantly with drugs metabolized by CYP2C19. This study highlights that, when they are further refined, in silico methods will provide rapid and reliable tools for predicting the propensity of any xenobiotics to inhibit P450-mediated metabolism.

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Authorship Contributions

Participated in research design: Salminen, Rahnasto-Rilla, Poso, Laitinen, Raunio, Lahtela-Kakkonen.

Conducted experiments: Salminen, Rahnasto-Rilla, Väänänen, Lahtela-Kakkonen.

Contributed new reagents or analytic tools: Imming, Meyer, Horling.

Performed data analysis: Salminen, Rahnasto-Rilla, Poso, Lahtela-Kakkonen.

Wrote or contributed to the writing of the manuscript: Salminen, Rahnasto-Rilla, Imming, Meyer, Poso, Raunio, Lahtela-Kakkonen.

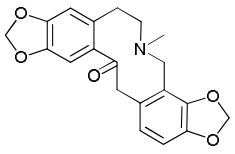
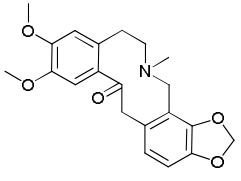
References

Allen FH (2002) The Cambridge Structural Database: a quarter of a million crystal structures and rising. *Acta Crystallogr B* **58**:380–388.
Berry LM and Zhao Z (2008) An examination of IC50 and IC50-shift experiments in assessing time-dependent inhibition of CYP3A4, CYP2D6 and CYP2C9 in human liver microsomes. *Drug Metab Lett* **2**:51–59.
Boobis A, Watelet JB, Whomsley R, Benedetti MS, Demoly P, and Tipton K (2009) Drug interactions. *Drug Metab Rev* **41**:486–527.
Bruno IJ, Cole JC, Edgington PR, Kessler M, Macrae CF, McCabe P, Pearson J, and Taylor R (2002) New software for searching the Cambridge Structural Database and visualizing crystal structures. *Acta Crystallogr B* **58**:389–397.
Casida JE (1970) Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J Agric Food Chem* **18**:753–772.
Clark RD and Fox PC (2004) Statistical variation in progressive scrambling. *J Comput Aided Mol Des* **18**:563–576.
Correia MA and Ortiz de Montellano PR (2005) Inhibition of cytochrome P450 enzymes, in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed) pp 247–322, Kluwer Academic/Plenum Publishers, New York.
Cramer RD, Patterson DE, and Bunce JD (1988) Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J Am Chem Soc* **110**:5959–5967.
Fang ZZ, Zhang YY, Ge GB, Huo H, Liang SC, and Yang L (2010) Time-dependent inhibition (TDI) of CYP3A4 and CYP2C9 by noscapine potentially explains clinical noscapine-warfarin interaction. *Br J Clin Pharmacol* **69**:193–199.
Foti RS, Rock DA, Han X, Flowers RA, Wienkers LC, and Wahlstrom JL (2012) Ligand-based design of a potent and selective inhibitor of cytochrome P450 2C19. *J Med Chem* **55**: 1205–1214.
Franklin MR (1971) The enzymic formation of methylenedioxyphenyl derivative exhibiting an isocyanide-like spectrum with reduced cytochrome P-450 in hepatic microsomes. *Xenobiotica* **1**:581–591.
Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, and Perry JK, et al. (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* **47**:1739–1749.
Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC, and Mainz DT (2006) Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem* **49**:6177–6196.
Gleeson MP, Davis AM, Chohan KK, Paine SW, Boyer S, Gavaghan CL, Arnby CH, Kankkonen C, and Albertson N (2007) Generation of in-silico cytochrome P450 1A2, 2C9, 2C19, 2D6, and 3A4 inhibition QSAR models. *J Comput Aided Mol Des* **21**:559–573.
Golbraikh A, Shen M, Xiao Z, Xiao YD, Lee KH, and Tropsha A (2003) Rational selection of training and test sets for the development of validated QSAR models. *J Comput Aided Mol Des* **17**:241–253.
Gotti R (2011) Capillary electrophoresis of phytochemical substances in herbal drugs and medicinal plants. *J Pharm Biomed Anal* **55**:775–801.
Guengerich FP (2008) Cytochrome p450 and chemical toxicology. *Chem Res Toxicol* **21**:70–83.
Guo Y, Chen Y, Tan ZR, Klaassen CD, and Zhou HH (2012) Repeated administration of berberine inhibits cytochromes P450 in humans. *Eur J Clin Pharmacol* **68**:213–217.
Hagel JM and Facchini PJ (2013) Benzylisoquinoline alkaloid metabolism: a century of discovery and a brave new world. *Plant Cell Physiol* **54**:647–672.
Halgren TA, Murphy RB, Friesner RA, Beard HS, Frye LL, Pollard WT, and Banks JL (2004) Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J Med Chem* **47**:1750–1759.
Han YL, Yu HL, Li D, Meng XL, Zhou ZY, Yu Q, Zhang XY, Wang FJ, and Guo C (2011) In vitro inhibition of Huanglian [Rhizoma coptidis (L.)] and its six active alkaloids on six cytochrome P450 isoforms in human liver microsomes. *Phytother Res* **25**:1660–1665.
Izzo AA and Ernst E (2009) Interactions between herbal medicines and prescribed drugs: an updated systematic review. *Drugs* **69**:1777–1798.
Jordan SA, Cunningham DG, and Marles RJ (2010) Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment. *Toxicol Appl Pharmacol* **243**:198–216.
Komotar RJ, Zacharia BE, Valhora R, Mocco J, and Connolly ES, Jr (2007) Advances in vasospasm treatment and prevention. *J Neurol Sci* **261**:134–142.
Lewis DF (2004) Quantitative structure-activity relationships (QSARs) for substrates of human cytochromes P450 CYP2 family enzymes. *Toxicol In Vitro* **18**:89–97.
Lewis DF, Lake BG, Ito Y, and Dickins M (2006) Lipophilicity relationships in inhibitors of CYP2C9 and CYP2C19 enzymes. *J Enzyme Inhib Med Chem* **21**:385–389.
Meyer A and Imming P (2011) Benzylisoquinoline alkaloids from the papaveraceae: the heritage of Johannes Gadamer (1867-1928). *J Nat Prod* **74**:2482–2487.
Murray M (2012) Toxicological actions of plant-derived and anthropogenic methylenedioxyphenyl-substituted chemicals in mammals and insects. *J Toxicol Environ Health B Crit Rev* **15**: 365–395.
Nakajima M, Suzuki M, Yamaji R, Takashina H, Shimada N, Yamazaki H, and Yokoi T (1999) Isoform selective inhibition and inactivation of human cytochrome P450s by methylenedioxyphenyl compounds. *Xenobiotica* **29**:1191–1202.
Newman DJ and Cragg GM (2012) Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod* **75**:311–335.
NiceData Software (2005) *Traditional Chinese Medicines Database. Version 2.1*, CambridgeSoft Corporation, Cambridge, MA.
Ohlsson S, Holm L, Myrberg O, Sundström A, and Yue QY (2008) Noscapine may increase the effect of warfarin. *Br J Clin Pharmacol* **65**:277–278.
Panzer A, Joubert AM, Bianchi PC, Hamel E, and Seegers JC (2001) The effects of chelidonine on tubulin polymerisation, cell cycle progression and selected signal transmission pathways. *Eur J Cell Biol* **80**:111–118.

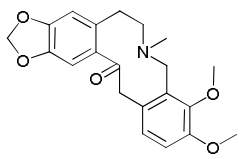
Pelkonen O, Turpeinen M, Hakkola J, Honkakoski P, Hukkanen J, and Raunio H (2008) Inhibition and induction of human cytochrome P450 enzymes: current status. *Arch Toxicol* **82**: 667–715.
Pelkonen O, Xu Q, and Fan TP (2014) Why is research on herbal medicinal products important and how can we improve its quality? *J Tradit Complement Med* **4**:1–7.
Posadzki P, Watson L, and Ernst E (2013) Herb-drug interactions: an overview of systematic reviews. *Br J Clin Pharmacol* **75**:603–618.
Rahnasto MK, Raunio HA, Wittekindt C, Salminen KA, Leppänen J, Juvonen RO, Poso A, and Lahtela-Kakkonen MK (2011) Identification of novel CYP2A6 inhibitors by virtual screening. *Bioorg Med Chem* **19**:7186–7193.
Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism data. *Drug Metab Rev* **34**:83–448.
Reynald RL, Sansen S, Stout CD, and Johnson EF (2012) Structural characterization of human cytochrome P450 2C19: active site differences between P450s 2C8, 2C9, and 2C19. *J Biol Chem* **287**:44581–44591.
Rosenborg S, Stenberg M, Otto S, Ostervall J, Masquelier M, Yue QY, Bertilsson L, and Eliasson E (2010) Clinically significant CYP2C inhibition by noscapine but not by glucosamine. *Clin Pharmacol Ther* **88**:343–346.
Salminen KA, Leppänen J, Venäläinen JI, Pasanen M, Auriola S, Juvonen RO, and Raunio H (2011a) Simple, direct, and informative method for the assessment of CYP2C19 enzyme inactivation kinetics. *Drug Metab Dispos* **39**:412–418.
Salminen KA, Meyer A, Jerabkova L, Korhonen LE, Rahnasto M, Juvonen RO, Imming P, and Raunio H (2011b) Inhibition of human drug metabolizing cytochrome P450 enzymes by plant isoquinoline alkaloids. *Phytomedicine* **18**:533–538.
Salminen KA, Meyer A, Imming P, and Raunio H (2011c) CYP2C19 progress curve analysis and mechanism-based inactivation by three methylenedioxyphenyl compounds. *Drug Metab Dispos* **39**:2283–2289.
Schrödinger (2011) *LigPrep, version 2.5*, Schrödinger, LLC, New York, NY.
Schrödinger (2012a) *Prime, version 3.0*, Schrödinger, LLC, New York, NY.
Schrödinger (2012b) *Glide, versions 3.0 and 5.8*, Schrödinger, LLC, New York, NY.
Schrödinger (2013) *Maestro, version 9.6*, Schrödinger, LLC, New York, NY.
Scordo MG, Melhus H, Stjernberg E, Edvardsson AM, and Wadelius M (2008) Warfarin-noscapine interaction: a series of four case reports. *Ann Pharmacother* **42**:448–450.
Scott SA, Sangkuhl K, Stein CM, Hulot JS, Mega JL, Roden DM, Klein TE, Sabatine MS, Johnson JA, and Shuldiner AR; Clinical Pharmacogenetics Implementation Consortium (2013) Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. *Clin Pharmacol Ther* **94**:317–323.
Singh D, Gupta R, and Saraf SA (2012) Herbs-are they safe enough? an overview. *Crit Rev Food Sci Nutr* **52**:876–898.
Stresser DM, Mao J, Kenny JR, Jones BC, and Grime K (2014) Exploring concepts of in vitro time-dependent CYP inhibition assays. *Expert Opin Drug Metab Toxicol* **10**:157–174.
Suzuki H, Kneller MB, Haining RL, Trager WF, and Rettie AE (2002) (+)-N-3-Benzyl-nirvanol and (-)-N-3-benzyl-phenobarbital: new potent and selective in vitro inhibitors of CYP2C19. *Drug Metab Dispos* **30**:235–239.
Suzuki H, Kneller MB, Rock DA, Jones JP, Trager WF, and Rettie AE (2004) Active-site characteristics of CYP2C19 and CYP2C9 probed with hydantoin and barbiturate inhibitors. *Arch Biochem Biophys* **429**:1–15.
Tripos International (2011) SYBYL-X 2.0, Tripos International, 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA.
Tsai HH, Lin HW, Simon Pickard A, Tsai HY, and Mahady GB (2012) Evaluation of documented drug interactions and contraindications associated with herbs and dietary supplements: a systematic literature review. *Int J Clin Pract* **66**:1056–1078.
Urniaż RD and Jóźwiak K (2013)X-ray crystallographic structures as a source of ligand alignment in 3D-QSAR. *J Chem Inf Model* **53**:1406–1414.
Vuddanda PR, Chakraborty S, and Singh S (2010) Berberine: a potential phytochemical with multispectrum therapeutic activities. *Expert Opin Investig Drugs* **19**:1297–1307.
Wang JF, Zhang CC, Chou KC, and Wei DQ (2009) Structure of cytochrome p450s and personalized drug. *Curr Med Chem* **16**:232–244.
Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4**:825–833.
Yao YM, Cao W, Cao YJ, Cheng ZN, Ou-Yang DS, Liu ZQ, and Zhou HH (2007) Effect of sinomenine on human cytochrome P450 activity. *Clin Chim Acta* **379**:113–118.

Address correspondence to: Kaisa A. Salminen, School of Pharmacy, Faculty of Health Sciences, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland. E-mail: kaisa.salminen@uef.fi

Table 6. Search results with information from the commercial TCM Database (NiceData 2005).

No. (IV)	Compound	Information gathered from the database	Number of plants* that contain this compound *More details; See Table 7
	Protopines		
1	Protopine 	Protopine Biflorine; Corydinine; Fumarine; Macleyine; 7-methyl-2,3:9,10-bis(methylenedioxy)-7,13a-secoberben-13a-one. [130-86-9] $C_{20}H_{19}NO_5$ (353.38). Antibacterial; antimalarial; antiasthmatic; choleretic (bile secretion promotor); smooth muscle relaxant; sedative; stops pregnancy in early stage (mus). **References** Jian Yin, et al., *Modern Study of Chinese Drugs and Clinical Applications (1),* Xueyuan Press, Beijing, 1993 (in Chinese). Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).	7
3	Cryptopine 	Cryptopine Cryptocavine. $C_{21}H_{23}NO_5$ (369.42). Hexa-prismatic or lamellar crystals (benzene), mp 220-221°C, 221-223°C. Similar action with narceine*; LD_{50} (mus ip) = 0.2mg/kg. *Antitussive; antihypertensive; promotes intestinal motion; respiratory stimulant. **References** Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese). Jiwu Wang, et al., *Handbook of Effective Components in Vegetal Medicines*, People Health Press, Beijing, 1986 (in Chinese).	11

4 Allocryptopine



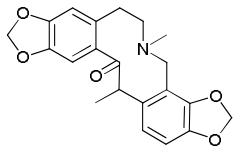
Allocryptopine [485-91-6] $C_{21}H_{23}NO_5$ (369.42). mp 163°C. Antiarrhythmic; antibacterial (*Staphylococcus* sp.); oxytocic. 4

References

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

6 Corycavamine



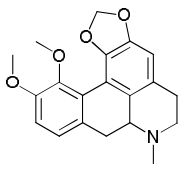
Corycavine [521-85-7] $C_{21}H_{21}NO_5$ (367.41). mp (±) 218-219°C. 1

Reference

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Aporphines

11 Bulbocapnine-methylether

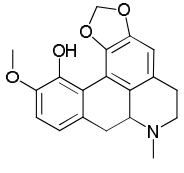


O-Methylbulbocapnine $C_{20}H_{21}NO_4$ (339.39). 1

References

Sheng-The Lu, et al., *Journal of Pharmaceutical Society* (*Japan*), 1972, 92 (7), 910.

12 Bulbocapnine



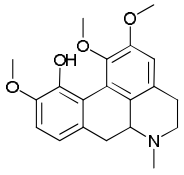
Bulbocapnine [298-45-3] $C_{19}H_{19}NO_4$ (325.37). Anticholinergic; anti-gastrin; dopamine receptor antagonist (in CNS); causes tetanic coma; inhibits excitation of striatum adenyl cyclase caused by dopamine; inhibits small intestinal movement *in vitro*; sedative; uterine stimulant (gpg and rbt); synergist of hypnotics; vasodilator; LD_{50} (mus, giving drug in rib) = 195mg/kg. 10

References

Editing Group of the Handbook of Bio-activity Components from Medicinal Plants, *Handbook of Bio-activity Components from Medicinal Plants*, The People"'s Medical Publishing House, Beijing, 1986 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

13 Isocorydine



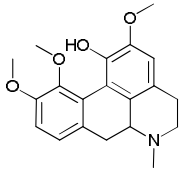
Isocorydine [475-67-2] $C_{20}H_{23}NO_4$ (341.41). mp 185-186°C. Adrenergic antagonist; antiarrhythmic (animal model); increases coronary flow and cerebral blood flow; LD_{50} (rat ip) = 10.9mg/kg. 11

References

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

14 Corydine



Corydine [476-69-7] $C_{20}H_{23}NO_4$ (341.41). mp (+) 149°C, (-) 149°C, (±) 165-167°C. Antineoplastic. 7

References

Yubin Ji, et al., *Pharmacological Action and Application of Available Antitumor Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1998 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

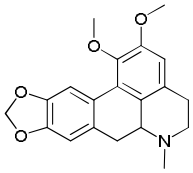
Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

15 Nantenine

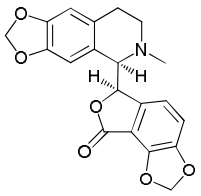
O-Methyl domesticine $C_{20}H_{21}NO_4$ (339.39). mp (+) 138-139°C. 3

References

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).



Phthalideisoquinoline

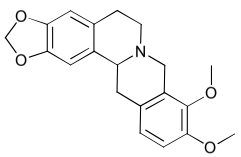
17	Capnoidine (Adlumidine) 	Adlumidine Bicuculline. [550-49-2] $C_{20}H_{17}NO_6$ (367.36). Orthogonal lamellar crystals (chloroform-methanol), mp 236-238°C; 215, [α] $_D^{25}$ = +116.2° (c = 22, chloroform), almost insoluble in water, very slightly soluble in alcohol, ether and ethane. gamma-aminobutyric acid antagonist; eclamptogenic, acts violently, attacks quickly and persistently; uterine stimulant.	7

References

Editing Group of the Handbook of Bio-activity Components from Medicinal Plants, *Handbook of Bio-activity Components from Medicinal Plants*, The People"'s Medical Publishing House, Beijing, 1986 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Protoberberines

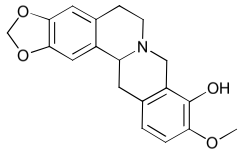
20	Canadine 	Canadine [522-97-4] $C_{20}H_{21}NO_4$ (339.39). mp (+) 132°C, (-) 134°C. Antibacterial; antihypertensive.	7

References

Editing Group of the Handbook of Bio-activity Components from Medicinal Plants, *Handbook of Bio-activity Components from Medicinal Plants*, The People"'s Medical Publishing House, Beijing, 1986 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

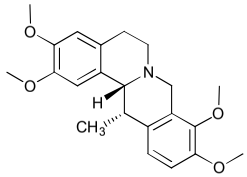
21	Nandinine	Nandinine $C_{19}H_{19}NO_4$ (325.37). mp 146°C.	2



Reference
Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

22 Corydaline

(+)-Corydaline [518-69-4] $C_{22}H_{27}NO_4$ (369.46). mp (+) 135°C. 1

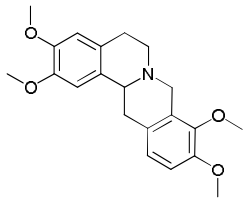


References
Jian Yin, et al., *Modern Study of Chinese Drugs and Clinical Applications (1),* Xueyuan Press, Beijing, 1993 (in Chinese).

Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

23 Tetrahydropalmatine

Tetrahydropalmatine Caseanine; Corydalis; Hyndarine; Rotundine. [2934-97-6] $C_{21}H_{25}NO_4$ (355.44). mp (+) 143°C, (-) 141-142°C, (±) 148°C. 6



References
Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

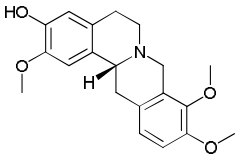
Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Chinese Materia Medica Editing Committee of the National Chinese Medicine and Pharmacology Bureau, *Chinese Materia Medica* ("*ZHONG HUA BEN CAO*"), Vol. 1 - Vol. 30, Shanghai Science and technology Press, Shanghai, 1999 (in Chinese).

24 Corypalmine

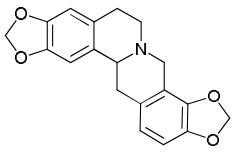
Corypalmine Tetrahydrojatrorrhizine. [6018-40-2] $C_{20}H_{23}NO_4$ (341.41). mp (+) 235-236°C, (-) 230°C, (±) 215-217°C. 4

References
Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).



Chinese Materia Medica Editing Committee of the National Chinese Medicine and Pharmacology Bureau, *Chinese Materia Medica* ("*ZHONG HUA BEN CAO*"), Vol. 1 - Vol. 30, Shanghai Science and technology Press, Shanghai, 1999 (in Chinese).

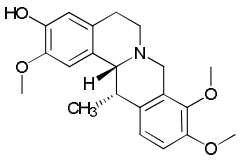
25 Stylopine



Stylopine $C_{19}H_{17}NO_4$ (323.35). mp (-) 204°C; (±) 222-223°C. 4

Reference
Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

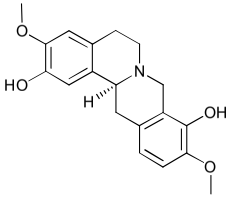
28 Corybulbine



(+)-Corybulbine $C_{21}H_{25}NO_4$ (355.44). 1

References
Jian Yin, et al., *Modern Study of Chinese Drugs and Clinical Applications (1),* Xueyuan Press, Beijing, 1993 (in Chinese).

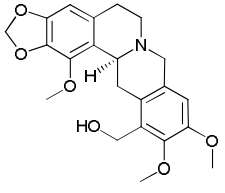
29 Scoulerine



Scoulerine (-)-Scoulerine; (*S*)-(-)-Scoulerine; (*S*)-Scoulerine; *L*-Scoulerin; (*S*)-5,8,13,13a-Tetrahydro-3,10-dimethoxy-6H-dibenzo[a,g]quinolizine-2,9-diol. [6451-73-6] $C_{19}H_{21}NO_4$ (327.38). mp (+) 197°C, (-) 204°C. 5

Reference
Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

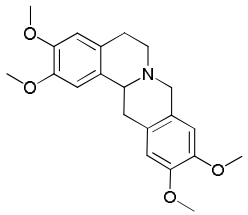
30 Mecambridine



Mecambridine [31098-60-9] $C_{22}H_{25}NO_6$ (399.45). Analgesic (mus); CNS depressant (mus). 1

Reference
Songyu Liu, et al., *Zhongyao Tongbao*, 1986, 11 (6), 360.

31 2,3,10,11-Tetramethoxy-berbine



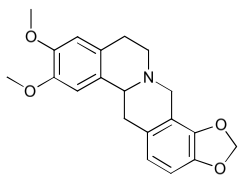
Xylopinine $C_{21}H_{25}NO_4$ (355.44). Crystals (ethanol), mp 181-182°C, $[\alpha]_D^{15}$ = -177.2° (c = 4.07, chloroform). Adrenergic α-receptor blocker; Hypnotic synergism with chloral hydrate (rat, 20mg/kg); LD_{50} (mus sc) = 108mg/kg, LD_{50} (mus iv) = 51.5mg/kg.

References

Jiwu Wang, et al., *Handbook of Effective Components in Vegetal Medicines*, People Health Press, Beijing, 1986 (in Chinese).

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

36 2,3-Dimethoxy-9,10-methylenedioxyberbine

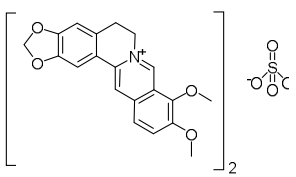


Sinactine $C_{20}H_{21}NO_4$ (339.39). mp (-) 175°C, (±) 168°C. 2

Reference

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

43 Berberine hemisulfate



Berberine Umbellatine. [2086-83-1] $C_{20}H_{18}NO_4$ (336.37). Adrenaline α_1, α_2 receptor agonist; analgesic; antidiarrheal; anti-inflammatory; antihypertensive; antimicrobial; antiprotozoal; antipyretic; choleretic; extends sleeping time due to pentobarbital; hypoglycemic; increases tolerance to anoxia; local anesthetic; reduces intra-ocular pressure in rbt; vasodilator, vascular smooth muscle relaxant; smooth muscle stimulant (uterus, bladder, gastrointestinal tract and bronchus); sedative. 9

References

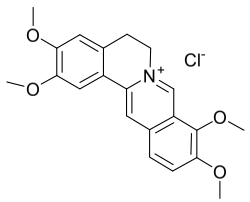
Editing Group of the Handbook of Bio-activity Components from Medicinal Plants, *Handbook of Bio-activity Components from Medicinal Plants*, The People"'s Medical Publishing House, Beijing, 1986 (in Chinese).

Jian Yin, et al., *Modern Study of Chinese Drugs and Clinical Applications (1),* Xueyuan Press, Beijing, 1993 (in Chinese).

Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

Baoyuan Jin, et al., *CCMM*, 1993, 18 (11), 675.
Chinese Materia Medica Editing Committee of the National Chinese Medicine and Pharmacology Bureau, *Chinese Materia Medica* ("*ZHONG HUA BEN CAO*"), Vol. 1 - Vol. 30, Shanghai Science and technology Press, Shanghai, 1999 (in Chinese).

44 Palmatine



Palmatine Berbericinine. [3486-67-7] $C_{21}H_{22}NO_4$ (352.41). mp 198-201°C. Analgesic; antiarrhythmic; antibacterial (*Staphylococcus aureus* and *Sporothrix* sp., EC = 500μg/ml, *Bacillus dysenteriae*, *B. coli*, β-*Streptococcus* and 12 kinds of molds); antifungal (*Candida albicans*, EC = 250μg/ml); antiviral (Asia α-Influenza virus); enhances myocardial contractility; cholinesterase inhibitor; kills Lewis-trypanosome (iodide); reduces area of myocardial infarction (rbt *in vivo*, chloride 0.75mg/kg, iv); LD_{50} (mus iv, sulfocyanate) = 98μg/kg. 18

References

Jian Yin, et al., *Modern Study of Chinese Drugs and Clinical Applications (1),* Xueyuan Press, Beijing, 1993 (in Chinese).

Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

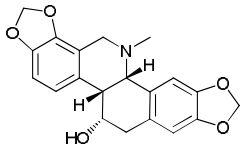
Youying Guo, et al., *APS*, 1998, 33 (5), 350.

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

Chinese Materia Medica Editing Committee of the National Chinese Medicine and Pharmacology Bureau, *Chinese Materia Medica* ("*ZHONG HUA BEN CAO*"), Vol. 1 - Vol. 30, Shanghai Science and technology Press, Shanghai, 1999 (in Chinese).

Others

45 Chelidonine



Chelidonine Diphyline; *D*-Chelidonine; Stylophorin. [476-32-4] $C_{20}H_{19}NO_5$ (353.38). mp 136-140°C, bp 220°C. Antibacterial; antispasmodic (smooth muscle); antiviral; cytotoxic (HeLa, ED_{50} = 0.27μg/ml, S_{180} and EAC); inhibits cardiac muscles (slows heart rate and stops beating in period of expansion in high dose); CNS depressant (sedative and hypnotic); inhibits mitosis (fibrocyte *in vitro*, 2.5×10^{-6} mol/L); inhibits skeletal muscles; acaricide; paralyses sensory and motor nerve; LD_{50} (mus iv) = 34.6±2.44mg/kg. 5

References

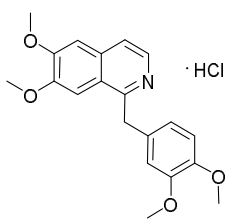
Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Yanjun Zhang, et al., *CCMM*, 1997, 22 (9), 550.

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

46 Papaverine hydrochloride



Papaverine [58-74-2] $C_{20}H_{21}NO_4$ (339.39). mp 147°C. Antineoplastic; antitussive; choleretic; platelet aggregation inhibitor; smooth muscle relaxant (hmn, dog); LD_{50} (mus iv) = 46.3mg/kg, (rat orl) = 750mg/kg, (mus orl) = 528mg/kg. 5

References

Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

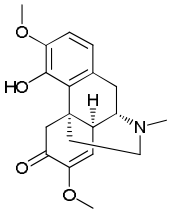
Yubin Ji, et al., *Pharmacological Action and Application of Available Antitumor Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1998 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and

technology Press, Shanghai, 1979 (in Chinese).

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

47 Sinomenine



Sinomenine Coculine; Cucoline; Kukoline. [115-53-7] $C_{19}H_{23}NO_4$ (329.40). mp 162°C. Analgesic (mus, rbt); antiarrhythmic (*in vitro* atrium of gpg); anti-inflammatory (rat, arthritis model due to methanol or egg white); antitussive (mus, cat); inhibits intestinal smooth muscle (*in vitro*); antihypertensive (dog, cat and rat, iv and orl); negative chronotropic action; releases histamine; LD_{50} (mus orl) = 580mg/kg, (111ujube) = 535mg/kg, (111ujube) = 285mg/kg, (dog orl) = 45mg/kg, (monkey orl) = 95mg/kg. 3

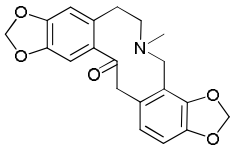
References

Yubin Ji, et al., *Pharmacological Action and Application of Available Composition of Traditional Chinese Medicine*, Heilongjiang Science and technology Press, Heilongjiang, 1995 (in Chinese).

Edited by Jiangsu New Medical College, *Chinese Medicine Dictionary*, Shanghai Science and technology Press, Shanghai, 1979 (in Chinese).

Wenji Sun, et al., *Brief Handbook of Natural Active Compounds*, Medicinal Science and Technology Press of China, Beijing, 1998 (in Chinese).

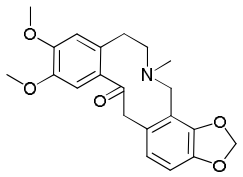
Table 7. The following 26 alkaloid were related to numerous medicinal plants. Search results from the commercial TCM Database (NiceData 2005).

No. (IV)	Compound	Information gathered from the database
	Protopines	
1	Protopine 	T4629 DA ZAO; Chinese Date (Equivalent Species: WU CI ZAO) Origin: *Ziziphus 112ujube* (Rhamnaceae) Part: ripe fruit. Effects: To supplement center and boost *qi,* nourish blood and quiet spirit. Indications: Reduced food intake due to spleen vacuity, lack of strength and sloppy stool, visceral agitation in women. Pharmacological: Antineoplastic, antimutagenic, hepatoprotective, anti-stress, enhances phago-function of mononuclear macrophages, anti-inflammatory, antiallergic. T3158 YING SU; Opium Poppy Origin: *Papaver somniferum* (Papaveraceae) Part: seed. Effects: To fortify spleen and promote digestion, clear heat and disinhibit urine. Indications: Stomach reflux, abdominal pain, diarrhea, prolapse of rectum. T2982 NAN TIAN ZHU ZI; Common Nandina Fruit Origin: *Nandina domestica* (Berberidaceae) Part: ripe fruit. Effects: To constrain lung and relieve cough, calm asthma. Indications: Enduring cough, asthma, pertussis. Pharmacological: Stimulates gastrointestinal smooth muscle. T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function. T1227 JIAN JU ZI JIN; Sharpspur Corydalis Origin: *Corydalis suaveolens* [Syn. *Corydalis sheareri*]. (Papaveraceae) Part: whole herb or tuber. Effects: To quicken blood and relieve pain, clear heat and resolve toxin. Indications: Stomachache, abdominal pain and diarrhea, knocks and falls, swelling toxin of welling abscess and sore, red eyes with gall. T1203 XIA TIAN WU; Decumbent Corydalis Origin: *Corydalis decumbens* [Syn. *Corydalis amabilis*]. (Papaveraceae) Part: rhizome. Effects: To move *qi* and quicken blood, free network vessels and relieve pain. Indications: Pain in deep tissues, neuralgia, swelling pain from fracture, hypertension and hemiplegia, sequel of

poliomyelitis, sciatica, rheumatic arthritis, knocks and falls. Pharmacological: Analgesic.

T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough, jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.

3 Cryptopine



T3159 YING SU KE; Opium Poppy Pericarp Origin: *Papaver somniferum* (Papaveraceae) Part: capsule. Effects: To constrain lung, astringe intestines, relieve pain. Indications: Enduring cough, chronic diarrhea, prolapse of rectum, pain in stomach duct and abdomen.

T3158 YING SU; Opium Poppy Origin: *Papaver somniferum* (Papaveraceae) Part: seed. Effects: To fortify spleen and promote digestion, clear heat and disinhibit urine. Indications: Stomach reflux, abdominal pain, diarrhea, prolapse of rectum.

T3157 YA PIAN; Opium Origin: *Papaver sommiferum* (Papaveraceae) Part: latex from unripe capsules. Effects: To relieve pain, astringe intestines, suppress cough. Indications: Pain in heart and abdomen, chronic diarrhea, chronic dysentery, cough without phlegm. Pharmacological: Antihypertensive, inhibits smooth muscles as antispasmodic.

T2311 CHUN HE QING HUA; Vernal Hylomecon* Origin: *Hylomecon vernalis* (Papaveraceae)

T2310 HE QING HUA; Japanese Hylomecon Origin: *Hylomecon japonica* (Papaveraceae) Part: root. Effects: To dispel wind and free network vessels, dissipate stasis and disperse swelling. Indications: Wind-damp impediment pain, knocks and falls.

T1964 XIAO YE QIU GUO ZI JIN; Fine-leaved Fumitory Origin: *Fumaria parviflora* (Papaveraceae)

T1963 YAO YONG QIU GUO ZI JIN; Medicinal Fumaria Origin: *Fumaria officinalis* (Papaveraceae)

T1524 HE BAO MU DAN GEN; Showy Bleedingheart Root Origin: *Dicentra spectabilis* (Papaveraceae) Part: rhizome. Effects: To dispel wind, quicken blood, settle pain. Indications: Incised wound, sore toxin, stomachache.

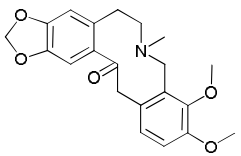
T1215 JU HUA HUANG LIAN; Yellowflower Corydalis Origin: *Corydalis pallida* (Papaveraceae) Part: root. Effects:

To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Welling abscess and boil, innominate toxin swelling

T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough, jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.

T429 JI YING SU; Mexican Pricklepoppy Origin: *Argemone 114ujube114e* (Papaveraceae) Part: whole herb. Effects: To effuse sweat and disinhibit water, clear heat and resolve toxin, relieve pain and itch. Indications: Common cold without sweating, jaundice, strangury, edema, eyelid laceration, mounting *qi*, scab and *lai,* syphilis. Pharmacological: Antiarrhythmic, antifungal, antiviral, antibacterial.

4 Allocryptopine



T2751 BO LUO HUI; Pink Plumepoppy Origin: *Macleaya cordata* (Papaveraceae) Part: herb with root. Effects: To dispel wind, dissipate stasis, resolve toxin, relieve pain, kill worms. Indications: Ulcer of uterine cervix, carcinoma of uterine cervix, carcinoma of thyroid, welling abscess and swelling of clove, ulcer of lower limb, hemorrhoids, eczema, snake or insect bites, painful swelling from knocks and falls, pain in joints due to rheumatalgia, decayed toothache, intractable lichen, trichomoniasis, brandy nose. Pharmacological: Trichomonacidal, antifungal, antibacterial.

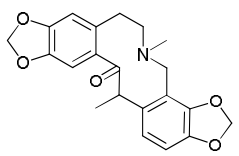
T2310 HE QING HUA; Japanese Hylomecon Origin: *Hylomecon japonica* (Papaveraceae) Part: root. Effects: To dispel wind and free network vessels, dissipate stasis and disperse swelling. Indications: Wind-damp impediment pain, knocks and falls.

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough, jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological:

Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.

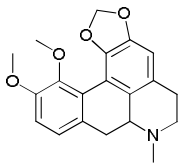
6 Corycavamine



T1206 ZI HUA YU DENG CAO; Incised Corydalis Origin: *Corydalis incisa* (Papaveraceae) Part: herb or root. Effects: To resolve toxin and kill worms, relieve pain. Indications: Toxin swelling of sores, scab and *lai,* intractable lichen, eczema, poisonous snake bite. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antimalarial, antibacterial.

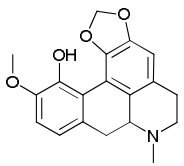
Aporphines

11 Bulbocapnine-methylether



T2625 HEI KE NAN; Largeleaf Spicebush Origin: *Lindera megaphylla* (Lauraceae) Part: root, bark or branch. Effects: To dispel wind and eliminate damp, warm center and move *qi,* disperse swelling and relieve pain. Indications: Wind-damp impediment pain, impediment pain numbness, cold pain in stomach duct and abdomen, mounting *qi*, sore swollen throat, lichen sore.

12 Bulbocapnine



T2079 MEI LI HAI YING SU; Beautiful Hornpoppy* Origin: *Glaucium pulchrum* (Papaveraceae)

T2077 HUANG HAI YING SU; Yellow Hornpoppy* Origin: *Glaucium flavum* (Papaveraceae)

T1963 YAO YONG QIU GUO ZI JIN; Medicinal Fumaria Origin: *Fumaria officinalis* (Papaveraceae)

T1215 JU HUA HUANG LIAN; Yellowflower Corydalis Origin: *Corydalis pallida* (Papaveraceae) Part: root. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Welling abscess and boil, innominate toxin swelling

T1210 MA CHANG LI ZI JIN; Marschall Corydalis * Origin: *Corydalis marschalliana* (Papaveraceae)

T1203 XIA TIAN WU; Decumbent Corydalis Origin: *Corydalis decumbens* [Syn. *Corydalis amabilis*]. (Papaveraceae) Part: rhizome. Effects: To move *qi* and quicken blood, free network vessels and relieve pain. Indications: Pain in deep tissues, neuralgia, swelling pain from fracture, hypertension and hemiplegia, sequel of poliomyelitis, sciatica, rheumatic arthritis, knocks and falls. Pharmacological: Analgesic.

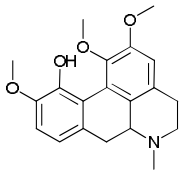
T1200 AO XIAN ZI JIN; Bulbous Corydalis Origin: *Corydalis cava* (Papaveraceae)

T1199 GAO JIA SUO ZI JIN; Caucasian Corydalis* Origin: *Corydalis caucasica* (Papaveraceae)

T1198 SHAN YAN HU SUO; Bird-in-a-bush Origin: *Corydalis bulbosa* [Syn. *Corydalis solida*]. (Papaveraceae)

T1198 SHAN YAN HU SUO; Bird-in-a-bush Origin: *Corydalis bulbosa* [Syn. *Corydalis solida*]. (Papaveraceae)

13 Isocorydine



T4308 TAI WAN TANG SONG CAO; Taiwan Meadowrue Origin: *Thalictrum urbainii* (Ranunculaceae)

T4117 BAI XIAN SHU; Shortstamen Stephania* Origin: *Stephania brachyandra* (Menispermaceae) Part: tuberoid. Effects: To move *qi* and quicken blood, dispel wind and relieve pain, clear heat and resolve toxin. Indications: Stomachache, wind-damp impediment pain, knocks and falls, dysmenorrhea, swelling toxin of welling abscess and boil, eczema.

T3148 LI CHUN HUA; Corn Poppy Origin: *Papaver commutatum* [Syn. *Papaver rhoeas*]. (Papaveraceae) Part: whole herb, flower or fruit. Effects: To suppress cough, settle pain, check diarrhea. Indications: Cough, migrainous headache, abdominal pain, dysentery. Pharmacological: Antineoplastic.

T2982 NAN TIAN ZHU ZI; Common Nandina Fruit Origin: *Nandina domestica* (Berberidaceae) Part: ripe fruit. Effects: To constrain lung and relieve cough, calm asthma. Indications: Enduring cough, asthma, pertussis. Pharmacological: Stimulates gastrointestinal smooth muscle.

T2776 JIAN YE SHI DA GONG LAO; Oregon-grape Origin: *Mahonia aquifolium* (Berberidaceae)

T2653 CHENG QIE ZI; Mountain Spicy Tree Origin: *Litsea cubeba* (Lauraceae) Part: fruit. Effects: To warm center and relieve pain, move *qi* and quicken blood, calm asthma, disinhibit urine. Indications: Chronic bronchitis, bronchial asthma, asthma, cold pain in stomach duct and abdomen, food accumulation and *qi* distention, stomach reflux vomiting, summerheat stroke with vomiting and diarrhea, diarrhea, cold mounting with abdominal pain, cold-damp water drum distention, inhibited urination, opacity of urine, toxin swelling of sores, toothache, cold-damp impediment pain, knocks and falls. Pharmacological: Antiasthmatic, anti-ischemia myocardial, platelet aggregation inhibitor, trichomonacidal, antifungal, antibacterial.

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*].

(Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1229 KUAI JING ZI JIN; Tuberous Corydalis* Origin: *Corydalis tuberosa* (Papaveraceae)

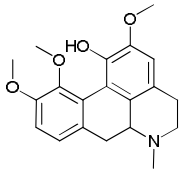
T1209 HUANG ZI JIN; Yellow Corydalis Origin: *Corydalis lutea* (Papaveraceae)

T1200 AO XIAN ZI JIN; Bulbous Corydalis Origin: *Corydalis cava* (Papaveraceae)

T467 XIANG YING ZHAO; Fragrant Tailgrape* Origin: *Artabotrys suaveolens* (Annonaceae)

14 Corydine

T4122 DING KE LA QIAN JIN TENG; Dinklage Stephania* Origin: *Stephania dinklagei* (Menispermaceae)



T2076 HAI YING SU; Ciliate Hornpoppy* Origin: *Glaucium fimbrilligerum* (Papaveraceae) Part: fruit. Effects: To settle pain and suppress cough. Indications: /

T2074 XIAO JIAO HAI YING SU; Black-spot Hornpoppy Origin: *Glaucium corniculatum* (Papaveraceae)

T1765 LUO BO HUA LING CAO; Lobb Poppy* Origin: *Eschscholzia lobbii* (Papaveraceae)

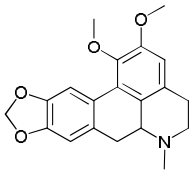
T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1218 CHI BAN YAN HU SUO; Toothedpetal Corydalis Origin: *Corydalis remota* [Syn. *Corydalis bulbosa* var. *117ujube117*]. (Papaveraceae) Part: various tuber. Effects: To quicken blood and dissipate stasis, move *qi* and relieve pain. Indications: Pain in lumbus and knees, pain in heart and abdomen, dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls.

T1210 MA CHANG LI ZI JIN; Marschall Corydalis * Origin: *Corydalis marschalliana* (Papaveraceae)

15 Nantenine

T2982 NAN TIAN ZHU ZI; Common Nandina Fruit Origin: *Nandina domestica* (Berberidaceae) Part: ripe fruit.



Effects: To constrain lung and relieve cough, calm asthma. Indications: Enduring cough, asthma, pertussis. Pharmacological: Stimulates gastrointestinal smooth muscle.

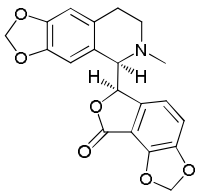
T2981 NAN TIAN ZHU GENG; Common Nandina Stem Origin: *Nandina domestica* (Berberidaceae) Part: branchlet. Effects: To clear damp heat, downbear counterflow *qi.* Indications: Damp-heat jaundice, diarrhea, heat strangury, red eyes with gall, cough and asthma, *ge* syndrome.

T2980 NAN TIAN ZHU GEN; Common Nandina Root Origin: *Nandina domestica* (Berberidaceae) Part: root. Effects: To clear heat, relieve cough, eliminate damp, resolve toxin. Indications: Lung heat cough, damp-heat jaundice, diarrhea, wind-damp impediment pain, sore, scrofula.

Phthalideisoquinoline

17 Capnoidine

(Adlumidine)



T1518 DOU ZHUANG HE BAO MU DAN; Dutchman's Breeches Origin: *Dicentra cucullaria* (Papaveraceae)

T1228 YAN HUANG LIAN; Rockliving Corydalis Origin: *Corydalis thalictrifolia* (Papaveraceae) Part: whole herb. Effects: To clear heat and resolve toxin, disinhibit damp, stanch bleeding and relieve pain. Indications: Hepatitis, oral ulcer, acute conjunctivitis nephelium, dysentery, abdominal pain and diarrhea, bleeding from hemorrhoids. Pharmacological: Antineoplastic, antibacterial.

T1219 TU YAN HU; Repent Corydalis* Origin: *Corydalis repens* var. *humosides* (Papaveraceae)

T1213 BIAN BING HUANG JIN; Flatstiped Corydalis Origin: *Corydalis mucronifera* (Papaveraceae) Part: whole herb with root. Effects: To clear heat and resolve toxin, relieve pain. Indications: Influenza, warm disease fever, gastritis, ulcer, dysentery, sciatica, swelling pain of sore and boil.

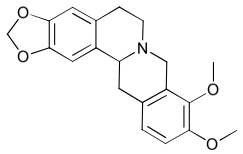
T1206 ZI HUA YU DENG CAO; Incised Corydalis Origin: *Corydalis incisa* (Papaveraceae) Part: herb or root. Effects: To resolve toxin and kill worms, relieve pain. Indications: Toxin swelling of sores, scab and *lai,* intractable lichen, eczema, poisonous snake bite. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antimalarial, antibacterial.

T1203 XIA TIAN WU; Decumbent Corydalis Origin: *Corydalis decumbens* [Syn. *Corydalis amabilis*]. (Papaveraceae) Part: rhizome. Effects: To move *qi* and quicken blood, free network vessels and relieve pain. Indications: Pain in deep tissues, neuralgia, swelling pain from fracture, hypertension and hemiplegia, sequel of poliomyelitis, sciatica, rheumatic arthritis, knocks and falls. Pharmacological: Analgesic.

T137 XUN ZHUANG SHAN YUAN CAO; Climbing Fumitory Origin: *Adlumia cirrhosa* [Syn. *Adlumia fungosa*]. (Papaveraceae)

Protoberberines

20 Canadine



T4618 DU HUA JIAO; Venenous Pricklyash* Origin: *Zanthoxylum veneficium* (Rutaceae)

T4593 DUAN CI HUA JIAO; Shortspine Pricklyash* Origin: *Zanthoxylum brachyacanthum* (Rutaceae)

T2308 BAI MAO GEN II; Golden-seal Origin: *Hydrastis canadensis* (Ranunculaceae)

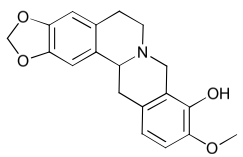
T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1221 YUAN YE SHAN WU GUI; Roundleaf Corydalis* Origin: *Corydalis rotundatour* (Papaveraceae)

T1201 HUA ZI JIN; Chinese Corydalis Origin: *Corydalis cheilanthifolia* (Papaveraceae)

T1200 AO XIAN ZI JIN; Bulbous Corydalis Origin: *Corydalis cava* (Papaveraceae)

21 Nandinine

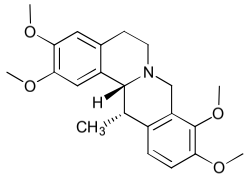


T2982 NAN TIAN ZHU ZI; Common Nandina Fruit Origin: *Nandina domestica* (Berberidaceae) Part: ripe fruit. Effects: To constrain lung and relieve cough, calm asthma. Indications: Enduring cough, asthma, pertussis. Pharmacological: Stimulates gastrointestinal smooth muscle.

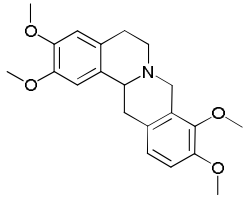
T2980 NAN TIAN ZHU GEN; Common Nandina Root Origin: *Nandina domestica* (Berberidaceae) Part: root. Effects: To clear heat, relieve cough, eliminate damp, resolve toxin. Indications: Lung heat cough, damp-heat jaundice, diarrhea, wind-damp impediment pain, sore, scrofula.

22 Corydaline

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.



23 Tetrahydropalmatine



T4128 JIN BU HUAN; Chinese Stephania Origin: *Stephania sinica* (Menispermaceae) Part: root. Effects: To clear heat and resolve toxin, dissipate stasis and relieve pain. Indications: stomachache, neuralgia, toothache, common cold, sore throat, diarrhea, dysentery, swelling toxin of welling abscess and flat abscess, wind-damp impediment pain, knocks and falls.

T3258 TU YE HUANG PI SHU; Glabrousleaf Chinese Corktree Origin: *Phellodendron chinense* var. *glabriusculum* (Rutaceae) Equivalent to HUANG BAI; *See* Amur Corktree.

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

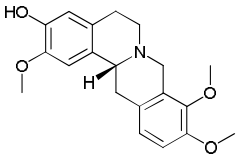
T1217 XIAO HUA HUANG JIN; Racemose Corydalis Origin: *Corydalis 120ujube120e* (Papaveraceae) Part: herb or root. Effects: To clear heat and disinhibit damp, resolve toxin and kill worms. Indications: Damp-heat diarrhea, dysentery, jaundice, red eyes with gall, otitis media, sore toxin, scab and lichen, poisonous snake bite. Pharmacological: Antihypertensive.

T1215 JU HUA HUANG LIAN; Yellowflower Corydalis Origin: *Corydalis pallida* (Papaveraceae) Part: root. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Welling abscess and boil, innominate toxin swelling

T1203 XIA TIAN WU; Decumbent Corydalis Origin: *Corydalis decumbens* [Syn. *Corydalis amabilis*]. (Papaveraceae) Part: rhizome. Effects: To move *qi* and quicken blood, free network vessels and relieve pain. Indications: Pain in deep tissues, neuralgia, swelling pain from fracture, hypertension and hemiplegia, sequel of poliomyelitis, sciatica, rheumatic arthritis, knocks and falls. Pharmacological: Analgesic.

24 Corypalmine

T3258 TU YE HUANG PI SHU; Glabrousleaf Chinese Corktree Origin: *Phellodendron chinense* var. *glabriusculum*



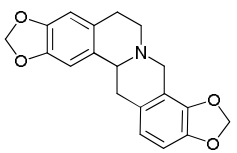
(Rutaceae) Equivalent to HUANG BAI; *See* Amur Corktree.

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1206 ZI HUA YU DENG CAO; Incised Corydalis Origin: *Corydalis incisa* (Papaveraceae) Part: herb or root. Effects: To resolve toxin and kill worms, relieve pain. Indications: Toxin swelling of sores, scab and *lai,* intractable lichen, eczema, poisonous snake bite. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antimalarial, antibacterial.

T629 TU HUANG LIAN; Wintergreen Barberry Origin: *Berberis julianae* (Berberidaceae) Part: root or herb. Effects: To clear heat and disinhibit damp, drain fire and resolve toxin. Indications: Damp-heat diarrhea dysentery, heat strangury, red eyes with gall, gum swelling, sore swollen throat, epidemic parotitis, erysipelas, eczema, heat toxin sores.

25 Stylopine



T2310 HE QING HUA; Japanese Hylomecon Origin: *Hylomecon japonica* (Papaveraceae) Part: root. Effects: To dispel wind and free network vessels, dissipate stasis and disperse swelling. Indications: Wind-damp impediment pain, knocks and falls.

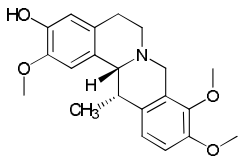
T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1215 JU HUA HUANG LIAN; Yellowflower Corydalis Origin: *Corydalis pallida* (Papaveraceae) Part: root. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Welling abscess and boil, innominate toxin swelling

T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough,

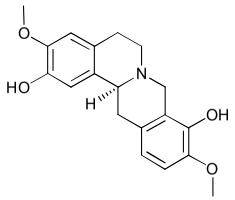
jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.

28 Corybulbine



T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

29 Scoulerine



T3157 YA PIAN; Opium Origin: *Papaver sommiferum* (Papaveraceae) Part: latex from unripe capsules. Effects: To relieve pain, astringe intestines, suppress cough. Indications: Pain in heart and abdomen, chronic diarrhea, chronic dysentery, cough without phlegm. Pharmacological: Antihypertensive, inhibits smooth muscles as antispasmodic.

T1524 HE BAO MU DAN GEN; Showy Bleedingheart Root Origin: *Dicentra spectabilis* (Papaveraceae) Part: rhizome. Effects: To dispel wind, quicken blood, settle pain. Indications: Incised wound, sore toxin, stomachache.

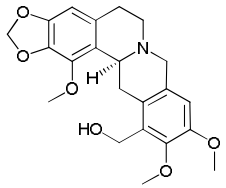
T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

T1215 JU HUA HUANG LIAN; Yellowflower Corydalis Origin: *Corydalis pallida* (Papaveraceae) Part: root. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Welling abscess and boil, innominate toxin swelling

T1206 ZI HUA YU DENG CAO; Incised Corydalis Origin: *Corydalis incisa* (Papaveraceae) Part: herb or root. Effects: To resolve toxin and kill worms, relieve pain. Indications: Toxin swelling of sores, scab and *lai,* intractable lichen, eczema, poisonous snake bite. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antimalarial, antibacterial.

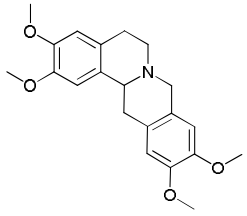
30 Mecambridine

T2834 HONG HUA LU RONG HAO; Redflower Meconopsis Origin: *Meconopsis punicea* (Papaveraceae) Part: whole herb with flower. Effects: To clear heat and resolve toxin, disinhibit damp, relieve pain. Indications: Ardent fever, tuberculosis, pneumonia, hepatitis, dysmenorrhea, leukorrhea, damp-heat edema, headache,



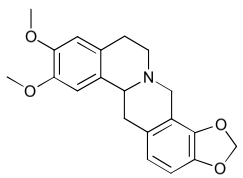
hypertension.

31 2,3,10,11-Tetramethoxy-berbine



T4130 HUANG YE DI BU RONG; Greenyellow Stephania Origin: *Stephania viridiflavens* (Menispermaceae) Equivalent to HE BAO DI BU RONG; *See* Dicentrine Stephania*.
T4120 HE BAO DI BU RONG; Dicentrine Stephania (Equivalent Species: HUANG YE DI BU RONG) Origin: *Stephania dicentrinifera* (Menispermaceae) Part: tuberoid. Effects: To dissipate stasis and relieve pain, clear heat and resolve toxin. Indications: Stomachache, dysentery, sore pharynx, knocks and falls, swollen welling abscess, sore and boil, poisonous snake bite. Pharmacological: Relaxes striated muscles.

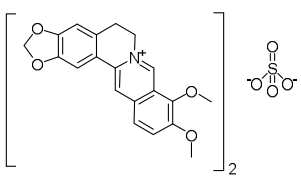
36 2,3-Dimethoxy-9,10-methylen-edioxyberbine



T4005 QING FENG TENG; Orientvine Origin: *Sinomenium acutum* (Menispermaceae) Part: stem. Effects: To dispel wind and free network vessels, eliminate damp and relieve pain. Indications: Wind-damp impediment pain, pain from arthritis, swelling in joints, paralytic and pruritus. Pharmacological: Analgesic, antihypertensive, anti-ischemia myocardial, antiarrhythmic, calcium channel antagonist, stimulates gastrointestinal smooth muscle, relaxes striated muscles, reduces phago-function of mononuclear macrophages, anti-inflammatory, antiallergic, anti-rejection.

T3148 LI CHUN HUA; Corn Poppy Origin: *Papaver commutatum* [Syn. *Papaver rhoeas*]. (Papaveraceae) Part: whole herb, flower or fruit. Effects: To suppress cough, settle pain, check diarrhea. Indications: Cough, migrainous headache, abdominal pain, dysentery. Pharmacological: Antineoplastic.

43 Berberine hemisulfate



T4629 DA ZAO; Chinese Date (Equivalent Species: WU CI ZAO) Origin: *Ziziphus 123ujube* (Rhamnaceae) Part: ripe fruit. Effects: To supplement center and boost *qi,* nourish blood and quiet spirit. Indications: Reduced food intake due to spleen vacuity, lack of strength and sloppy stool, visceral agitation in women. Pharmacological: Antineoplastic, antimutagenic, hepatoprotective, anti-stress, enhances phago-function of mononuclear macrophages, anti-inflammatory, antiallergic.

T4118 BAI YAO ZI; Oriental Stephania Origin: *Stephania cepharantha* (Menispermaceae) Part: tuberoid. Effects: To clear heat and resolve toxin, dispel wind and relieve pain, cool blood and stanch bleeding.

Indications: Septicemia, acute hepatitis, bacillary dysentery, parotitis, neurodermatitis, sore swollen throat, heat toxin swollen welling abscess, wind-damp impediment pain, abdominal pain and diarrhea, blood ejection, bleeding, spontaneous external bleeding, bleeding due to external injury. Pharmacological: Enhances hematopoietic function, antibacterial, antiallergic.

T3256 HUANG BAI; Amur Corktree (Equivalent Species: HUANG PI SHU, TU YE HUANG PI SHU, TAI WAN HUANG BO) Origin: *Phellodendron amurense* (Rutaceae) Part: bark. Effects: To drain fire, dispel damp and resolve toxin. Indications: Damp-heat dysentery, tuberculosis, epidemic meningitis, acute conjunctivitis, trachoma, fever, abdominal pain, diarrhea, suppurative hematochezia, tenesmus, jaundice, yellow thick foul leukorrhagia, swelling pain in knees and feet, urinary tract infection, boil, sore, ulcer, eczema, mouth sore, hemorrhoids, burns, scalds, exuberance of fire with tidal fever, tidal fever with night sweat, emission. Pharmacological: Cardiotonic, antihypertensive, antiarrhythmic, enhances ability of anti-anoxia, platelet aggregation inhibitor, hypoglycemic, trichomonacidal, antiamebic, antimalarial, antifungal, antibacterial, anti-inflammatory, antiallergic, anti-rejection.

T2783 HUA NAN GONG LAO MU; Japanese Mahonia Origin: *Mahonia japonica* (Berberidaceae) Equivalent to SHI DA GONG LAO MU; *See* Leatherleaf Mahonia.

T1166 HUANG LIAN; Chinese Goldthread (Equivalent Species: SAN JIAO YE HUANG LIAN, E MEI YE HUANG LIAN, YUN NAN HUANG LIAN) Origin: *Coptis chinensis* (Ranunculaceae) Part: rhizome. Effects: To clear heat and dry damp, drain fire and resolve toxin, lower blood pressure. Indications: Febrile infectious diseases, dysentery, blood ejection, spontaneous external bleeding, diphtheria, infection of upper respiratory tract, scarlatina, typhoid fever, acute conjunctivitis, otitis media, acute surgical infection, septicemia, hepatitis, trichomoniasis, eruptive dermatitis, hypertension, vexation and agitation, delirious raving, damp-heat glomus in chest, damp-heat diarrhea dysentery, insomnia and vexation due to effulgent fire, stomach heat vomiting, swift digestion with rapid hungering, red eyes with gall due to liver fire, heat toxin sores, clove sore running yellow, gum swelling and pain, mouth sore, tongue sores, genital swelling, bleeding from hemorrhoids, eczema, scalds. Pharmacological: Antiarrhythmic, antihypertensive, coronary vasodilator, enhances ability of anti-anoxia, antineoplastic, platelet aggregation inhibitor, hypoglycemic, choleretic, stimulates gastrointestinal smooth muscle, antifungal, antiviral, antibacterial, immunoenhancer, enhances phago-function of mononuclear macrophages, anti-inflammatory.

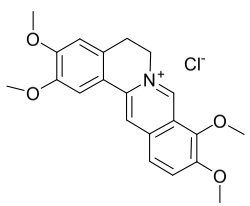
T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from

ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough, jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.
T637 JIN HUA XIAO BO; Wilson Barberry Origin: *Berberis wilsonae* (Berberidaceae) Part: root. Effects: To clear heat and dry damp, drain fire and resolve toxin. Indications: Bacillary dysentery, damp-heat diarrhea dysentery, infection of upper respiratory tract, lung heat cough, urinary tract infection, cholecystitis, leukopenic complications of carcinoma, jaundice, red eyes with gall, infant mouth sore, heat toxin swollen welling abscess.

T633 RI BEN XIAO BO; Japanese Barberry Origin: *Berberis thunbergii* (Berberidaceae) Part: root, root bark, stem and leaf. Effects: To clear heat and dry damp, drain fire and resolve toxin. Indications: Damp-heat diarrhea dysentery, stomach heat pain, red eyes with gall, mouth sore, sore swollen throat, acute eczema, scalds.

T629 TU HUANG LIAN; Wintergreen Barberry Origin: *Berberis julianae* (Berberidaceae) Part: root or herb. Effects: To clear heat and disinhibit damp, drain fire and resolve toxin. Indications: Damp-heat diarrhea dysentery, heat strangury, red eyes with gall, gum swelling, sore swollen throat, epidemic parotitis, erysipelas, eczema, heat toxin sores.

44 Palmatine



T2780 XI YE GONG LAO MU; Chinese Mahonia Origin: *Mahonia 125ortune* (Berberidaceae) Equivalent to SHI DA GONG LAO MU; *See* Leatherleaf Mahonia.

T631 XI YE XIAO BO; Poiret Barberry Origin: *Berberis poiretii* (Berberidaceae) Part: root, stem and bark. Effects: To clear heat and dry damp, drain fire and resolve toxin. Indications: Bacillary dysentery, damp-heat dysentery, diarrhea, jaundice, infection of upper respiratory tract, sore pharynx, urinary tract infection, cholecystitis, leukopenic complications of carcinoma, eczema, sores, mouth sore, red eyes. Pharmacological: Antihypertensive, anti-ischemia myocardial, antiarrhythmic, coronary vasodilator, calcium channel antagonist, peripheral vasodilator, antineoplastic, enhances hematopoietic function, leukogenic, platelet aggregation inhibitor, anticoagulative/antithrombotic, relaxes striated muscles, cholinesterase inhibitor, antimalarial, antifungal, antibacterial, enhances phago-function of mononuclear macrophages.

T633 RI BEN XIAO BO; Japanese Barberry Origin: *Berberis thunbergii* (Berberidaceae) Part: root, root bark, stem and leaf. Effects: To clear heat and dry damp, drain fire and resolve toxin. Indications: Damp-heat diarrhea dysentery, stomach heat pain, red eyes with gall, mouth sore, sore swollen throat, acute eczema, scalds.

T1166 HUANG LIAN; Chinese Goldthread (Equivalent Species: SAN JIAO YE HUANG LIAN, E MEI YE HUANG LIAN, YUN NAN HUANG LIAN) Origin: *Coptis chinensis* (Ranunculaceae) Part: rhizome. Effects: To clear heat and dry damp, drain fire and resolve toxin, lower blood pressure. Indications: Febrile infectious diseases, dysentery, blood ejection, spontaneous external bleeding, diphtheria, infection of upper respiratory tract, scarlatina, typhoid fever, acute conjunctivitis, otitis media, acute surgical infection, septicemia, hepatitis, trichomoniasis, eruptive dermatitis, hypertension, vexation and agitation, delirious raving, damp-heat glomus in chest, damp-heat diarrhea dysentery, insomnia and vexation due to effulgent fire, stomach heat vomiting, swift digestion with rapid hungering, red eyes with gall due to liver fire, heat toxin sores, clove sore running yellow, gum swelling and pain, mouth sore, tongue sores, genital swelling, bleeding from hemorrhoids, eczema, scalds. Pharmacological: Antiarrhythmic, antihypertensive, coronary vasodilator, enhances ability of anti-anoxia, antineoplastic, platelet aggregation inhibitor, hypoglycemic, choleretic, stimulates gastrointestinal smooth muscle, antifungal, antiviral, antibacterial, immunoenhancer, enhances phago-function of mononuclear macrophages, anti-inflammatory.

T1167 SAN JIAO YE HUANG LIAN; Deltoid Goldthread Origin: *Coptis deltoidea* (Ranunculaceae) Equivalent to HUANG LIAN; *See* Chinese Goldthread.

T1169 E MEI YE HUANG LIAN; Omei Mountain Goldthread Origin: *Coptis omeiensis* (Ranunculaceae) Equivalent to HUANG LIAN; *See* Chinese Goldthread.

T1170 YUN NAN HUANG LIAN; Yunnan Goldthread Origin: *Coptis teetoides* [Syn. *Coptis teeta*]. (Ranunculaceae) Equivalent to HUANG LIAN; *See* Chinese Goldthread.

T627 XIAO BO; Amur Barberry Origin: *Berberis amurensis* (Berberidaceae) Part: root and branchlet. Effects: To clear heat and dry damp, resolve toxin. Indications: Enteritis, dysentery, chronic cholecystitis, acute hepatitis, chronic hepatitis, bacillary dysentery, infection of upper respiratory tract, urinary tract infection, leukopenic complications of carcinoma, innominate toxin swelling, erysipelas, eczema, red eyes, mouth sore. Pharmacological: Antihypertensive, enhances hematopoietic function, choleretic, enhances phago-function of mononuclear macrophages.

T1895 TIAN XIAN TENG; Common Fibraurea Origin: *Fibraurea recisa* (Menispermaceae) Part: root, stem or leaf. Effects: To clear heat and resolve toxin, disinhibit damp. Indications: Acute tonsillitis, pharyngolaryngitis, infection of upper respiratory tract, conjunctivitis, jaundice, gastroenteritis, dysentery, child indigestion, food poisoning, salpingitis, acute endometritis, chronic endometritis, acute pelvic inflammation, vaginitis, sore and

boil, burns and scalds. Pharmacological: Antipyretic, antifungal.

T4288 MA WEI LIAN; Manyleaf Meadowrue (Equivalent Species: BEI JIA ER TANG SONG CAO, HUANG TANG SONG CAO) Origin: *Thalictrum foliolosum* (Ranunculaceae) Part: rhizome and root. Effects: To clear heat and dry damp, drain fire and resolve toxin. Indications: Influenza, fever in children, common cold with fever, measles, malaria, damp-heat diarrhea dysentery, jaundice, red eyes with gall. Pharmacological: Antihypertensive, antiarrhythmic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antibacterial.

T2781 XI YE GONG LAO YE; Chinese Mahonia Leaf Origin: *Mahonia 127ortune* (Berberidaceae) Equivalent to SHI DA GONG LAO YE; *See* Leatherleaf Mahonia Leaf.

T2783 HUA NAN GONG LAO MU; Japanese Mahonia Origin: *Mahonia japonica* (Berberidaceae) Equivalent to SHI DA GONG LAO MU; *See* Leatherleaf Mahonia.

T2784 HUA NAN GONG LAO YE; Japanese Mahonia Leaf Origin: *Mahonia japonica* (Berberidaceae) Equivalent to SHI DA GONG LAO YE; *See* Leatherleaf Mahonia Leaf.

T3256 HUANG BAI; Amur Corktree (Equivalent Species: HUANG PI SHU, TU YE HUANG PI SHU, TAI WAN HUANG BO) Origin: *Phellodendron amurense* (Rutaceae) Part: bark. Effects: To drain fire, dispel damp and resolve toxin. Indications: Damp-heat dysentery, tuberculosis, epidemic meningitis, acute conjunctivitis, trachoma, fever, abdominal pain, diarrhea, suppurative hematochezia, tenesmus, jaundice, yellow thick foul leukorrhagia, swelling pain in knees and feet, urinary tract infection, boil, sore, ulcer, eczema, mouth sore, hemorrhoids, burns, scalds, exuberance of fire with tidal fever, tidal fever with night sweat, emission. Pharmacological: Cardiotonic, antihypertensive, antiarrhythmic, enhances ability of anti-anoxia, platelet aggregation inhibitor, hypoglycemic, trichomonacidal, antiamebic, antimalarial, antifungal, antibacterial, anti-inflammatory, antiallergic, anti-rejection.

T3257 HUANG PI SHU; Chinese Corktree Origin: *Phellodendron chinense* (Rutaceae) Equivalent to HUANG BAI; *See* Amur Corktree.

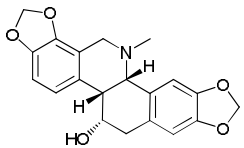
T3339 HAI SONG ZI; Korean Pine Seed Origin: *Pinus koraiensis* (Pinaceae) Part: seed. Effects: To moisten dryness, nourish blood, dispel wind. Indications: Dry cough due to lung dryness, vacuity constipation, joints wind, wind impediment. Pharmacological: Antiatherogenic.

T4130 HUANG YE DI BU RONG; Greenyellow Stephania Origin: *Stephania viridiflavens* (Menispermaceae) Equivalent to HE BAO DI BU RONG; *See* Dicentrine Stephania*.

T1230 YAN HU SUO; Yanhusuo Origin: *Corydalis yanhusuo* [Syn. *Corydalis turtschaninovii* f. *yanhusuo*]. (Papaveraceae) Part: rhizome. Effects: To quicken blood, disinhibit *qi,* relieve pain. Indications: Chest and rib-side pain, pain in stomach duct and abdomen, neuralgia, gastrointestinal spasm, amenorrhea and dysmenorrhea, postpartum stasis stagnation abdominal pain, painful swelling from knocks and falls. Pharmacological: Analgesic, antiarrhythmic, coronary vasodilator, enhances adrenal cortex function.

Others

45 Chelidonine



T4166 ER YE BAO YING SU; Celandine Poppy Origin: *Stylophorum diphyllum* (Papaveraceae)

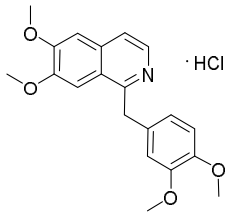
T3151 YE YING SU; Iceland Poppy (Equivalent Species: HEI SHUI YE YING SU) Origin: *Papaver nudicaule* (Papaveraceae) Part: fruit, shell or whole herb with flower. Effects: To constrain lung and relieve cough, astringe intestines and check diarrhea, settle pain. Indications: Enduring cough and asthma, diarrhea, hematochezia, prolapse of rectum, emission, vaginal discharge, headache, stomachache, dysmenorrhea. Pharmacological: Stimulates gastrointestinal smooth muscle.

T2310 HE QING HUA; Japanese Hylomecon Origin: *Hylomecon japonica* (Papaveraceae) Part: root. Effects: To dispel wind and free network vessels, dissipate stasis and disperse swelling. Indications: Wind-damp impediment pain, knocks and falls.

T1529 TU CHUANG HUA; Slenderstalk Dicranostigma Origin: *Dicranostigma franchetianum* [Syn. *Dicranostigma leptopodum*]. (Papaveraceae) Part: whole herb. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain, kill worms. Indications: Pain in throat, toothache, scrofula, bald sores, scab and lichen, welling abscess and boil, common wart. Pharmacological: Anti-ischemia cerebral, inhibits smooth muscles as antispasmodic.

T943 BAI QU CAI; Greater Celandine Origin: *Chelidonium majus* (Papaveraceae) Part: herb. Effects: To settle pain and relieve cough, disinhibit urine and resolve toxin. Indications: Pain in sensory nerve endings, pain from ulcer in digestive tract, stomachache, abdominal pain, enteritis, dysentery, chronic bronchitis, pertussis, cough, jaundice, edema, ascites, scab and lichen with swelling of sores, snake or insect bites. Pharmacological: Analgesic, antineoplastic, inhibits smooth muscles as antispasmodic, antifungal, antiviral, antibacterial.

46 Papaverine hydrochloride



T4118 BAI YAO ZI; Oriental Stephania Origin: *Stephania cepharantha* (Menispermaceae) Part: tuberoid. Effects: To clear heat and resolve toxin, dispel wind and relieve pain, cool blood and stanch bleeding. Indications: Septicemia, acute hepatitis, bacillary dysentery, parotitis, neurodermatitis, sore swollen throat, heat toxin swollen welling abscess, wind-damp impediment pain, abdominal pain and diarrhea, blood ejection, bleeding, spontaneous external bleeding, bleeding due to external injury. Pharmacological: Enhances hematopoietic function, antibacterial, antiallergic.

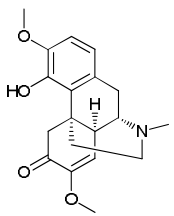
T3159 YING SU KE; Opium Poppy Pericarp Origin: *Papaver somniferum* (Papaveraceae) Part: capsule. Effects: To constrain lung, astringe intestines, relieve pain. Indications: Enduring cough, chronic diarrhea, prolapse of rectum, pain in stomach duct and abdomen.

T3158 YING SU; Opium Poppy Origin: *Papaver somniferum* (Papaveraceae) Part: seed. Effects: To fortify spleen and promote digestion, clear heat and disinhibit urine. Indications: Stomach reflux, abdominal pain, diarrhea, prolapse of rectum.

T3157 YA PIAN; Opium Origin: *Papaver sommiferum* (Papaveraceae) Part: latex from unripe capsules. Effects: To relieve pain, astringe intestines, suppress cough. Indications: Pain in heart and abdomen, chronic diarrhea, chronic dysentery, cough without phlegm. Pharmacological: Antihypertensive, inhibits smooth muscles as antispasmodic.

T840 FAN MU GUA; Papaya Fruit Origin: *Carica papaya* (Caricaceae) Part: fruit. Effects: To disperse food and promote lactation, eliminate damp and free network vessels, resolve toxin and expel worms. Indications: Stomachache, dysentery, inhibited urine and stool, wind impediment, foot rot. Pharmacological: Antihypertensive, peripheral vasodilator, antineoplastic, anticoagulative/antithrombotic, antifungal, antibacterial, antioxidant.

47 Sinomenine



T4005 QING FENG TENG; Orientvine Origin: *Sinomenium acutum* (Menispermaceae) Part: stem. Effects: To dispel wind and free network vessels, eliminate damp and relieve pain. Indications: Wind-damp impediment pain, pain from arthritis, swelling in joints, paralytic and pruritus. Pharmacological: Analgesic, antihypertensive, anti-ischemia myocardial, antiarrhythmic, calcium channel antagonist, stimulates gastrointestinal smooth muscle, relaxes striated muscles, reduces phago-function of mononuclear macrophages, anti-inflammatory, antiallergic, anti-rejection.

T2861 BIAN FU GE GEN; Asiatic Moonseed Root Origin: *Menispermum dauricum* (Menispermaceae) Part: rhizome. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain, disinhibit damp.

Indications: Sore swollen throat, lung heat cough, epidemic parotitis, diarrhea, jaundice, wind-damp impediment pain, swelling pain from hemorrhoids, snake or insect bites. Pharmacological: Antiarrhythmic, antihypertensive, platelet aggregation inhibitor, anticoagulative/antithrombotic, relaxes striated muscles, antibacterial, anti-inflammatory, antiallergic.

T2860 BIAN FU GE; Asiatic Moonseed Origin: *Menispermum dauricum* (Menispermaceae) Part: rattan. Effects: To clear heat and resolve toxin, disperse swelling and relieve pain. Indications: Lumbago, scrofula, sore swollen throat, diarrhea and dysentery, swelling pain from hemorrhoids.

KAISA SALMINEN

Alkaloid-containing plants have been used in human medicine for thousands of years. Today they still represent an important source of active pharmaceuticals. Alkaloids are also the potential active ingredients in many herbal preparations worldwide. The increasing use of herbal preparations has generated significant concern about harmful drug interactions. In this study, many isoquinoline alkaloids were found to evoke significant inhibition of the most important human drug-metabolizing CYP enzymes that is the most common cause of metabolism-based drug interactions.

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