Pharmacokinetics, pharmacodynamics and safety profile of novel non-steroidal androgen receptor antagonists FL 442 AB and FL 425 AB

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Saad Tanvir: Pharmacokinetics, pharmacodynamics and safety profile of novel non-steroidal androgen receptor antagonists FL 442 AB and FL 425 AB
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Prostate cancer is the second most common cancer worldwide in males and fifth most common cancer overall. Androgen deprivation therapy has been the standard treatment for prostate cancer. Androgen deprivation therapy involves surgical castration or medical castration. Medical castration is achieved by the use of LHRH agonist or antagonists. Medical castration combined with androgen receptor antagonists is called combined androgen blockade. Prostate cancer transforms to chemical castration resistant prostate cancer by the use of androgen deprivation therapy. Development of better treatment regime for prostate cancer is much needed.

Two novel non-steroidal androgen receptor antagonists FL 442 AB and its nitro analogue FL 425 AB were selected to evaluate their oxidative metabolism and inhibition potential aspects. Oxidative metabolism of FL 442 AB and FL 425 AB were studied in-vitro to check their stability profile and level of formed metabolites under CYP incubation conditions. Oxidative metabolites of FL 442 AB and FL 425 AB were evaluated in-vitro using LNCAP and VCAP prostate cancer cell cultures to check their antagonistic response. Three inhibition experiments were performed to find out the inhibition of hepatic coumarin 7-hydroxylation (CYP 2A6), 7-ethoxyresorufin O-deethylation (CYP 1A2), 7-benzyl 4-trifluoromethylcoumarin (CYP 3A4) by FL 442 AB and FL 425 AB and IC_{50} values were calculated. Results showed that FL 442 AB and FL 425 AB had anti androgenic activity and they were selective to androgen receptor (AR) and caused its inhibition. In-vitro data suggested that oxidative metabolites of FL 442 AB inhibited transcription of AR whereas oxidative metabolites of FL 425 AB were toxic. CYP 1A2 and CYP 2A6 were inhibited weakly (IC_{50}=330 µM and 140 µM respectively) by FL 442 AB whereas CYP 1A2 was inhibited weakly (IC_{50}=120 µM) by FL 425 AB. No inhibition of CYP 3A4 was observed by FL 442 AB and FL 425 AB. So pre-clinical pharmacology data suggests that novel non-steroidal androgen receptor antagonists FL 442 AB and FL 425 AB have shown good metabolic stability.

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Kuopio, September 2014

Saad Tanvir

LIST OF ABBREVIATIONS

AIS	Androgen insensitivity syndrome
AR	Androgen receptor
ADT	Androgen deprivation therapy
BFC	7-Benzyl 4-trifluoromethylcoumarin
CRPC	Castration resistant prostate cancer
CYP	Cytochrome P450
DBD	DNA binding domain
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
GC	Gas chromatography
HFC	7-Hydroxy-4-trifluoromethylcoumarin
HPLC	High performance liquid chromatography
IC_{50}	Concentration required for 50% inhibition
LBD	Ligand binding domain
MS	Mass spectrometry
NR	Nuclear receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NTD	N-Terminal regulatory domain
PSA	Prostate specific antigen

1. INTRODUCTION

The male sex hormones are called as androgens; androgen word originates from the two Greek words '*andros*', man, and '*gennan*', to produce. Testosterone and its active metabolite 5-α-dihydrotestosterone (DHT) are most important endogenous androgens. Testis Leydig cells produce 95% and adrenal gland produce 5% of testosterone. These hormones are involved in the penile and scrotum growth, body hair growth, thickness and darkness of skin, lower pitched voice (thickening of vocal cords), skeletal growth, prostate growth, stimulation and maintenance of sexual function in males (Bertram G. 2004).

The effects of androgens are mediated by androgen receptor (AR), free AR form complexes with chaperone proteins in cytosol when testosterone or 5-DHT binds to the AR in the cytosol; chaperone proteins releases the receptor, then the structure is altered and receptor hormone complex is taken up by the nucleus. Various factors contribute to the AR function including basal transcription machinery, chromatin remodeling and interactions with co-regulator proteins. The growth and spread of prostate cancer is dependent heavily on AR. So AR poses an important target for drugs against prostate cancer (Kaikkonen et al. 2009).

Androgen receptor antagonists are developed to control the AR activation and proliferation of prostate cancer. Two major types of AR antagonists are steroidal and non-steroidal. Prostate cancer is most often converted into castration resistant state after the treatment with existing non-steroidal AR antagonists and no longer beneficial to treat. So it's important to develop novel AR antagonists to inhibit AR effectively and to treat Prostate cancer efficiently (Chen et al. 2009; Poutiainen et al. 2012).

In the current work, the structure and physiological functions of AR, its relation with prostate cancer and existing AR antagonists were reviewed in detail under literature review. Oxidative metabolism of two novel non-steroidal AR antagonists FL 442 AB and FL425 AB were studied in-vitro under microsomal CYP incubation conditions to compare their stability and metabolites between mouse and human liver microsomes. Oxidative metabolites of FL 442 AB and FL 425 AB were also studied for their AR antagonistic response in-vitro. Three inhibition experiments were designed to study the inhibition of hepatic coumarin 7-hydroxylation (CYP 2A6), CYP 1A2 and CYP 3A4 by FL 442 AB and FL 425 AB and to calculate IC50 values of these ligands.

2. LITERATURE REVIEW

2.1 Androgen receptor

Specific effects of androgens are managed by androgen receptor (AR), which is ligand activated transcription factor, and a part of nuclear receptor (NR) proteins super family (Tsai & O'Malley 1994; Poutiainen et al. 2012). There are three classes of nuclear receptor (NR) super family; class I contains glucocorticoid, mineralocorticoid, estrogen, progesterone and androgen receptors, Class II contains peroxisome proliferator, farnesoid, liver oxysterol, and retinoid receptors and hybrid class consists of thyroid and vitamin D receptors. Androgen receptors (AR) belong to the class I of Nuclear Receptor (NR) super family (Rang et al. 2011).

2.1.1 Structure and functional regions

Nuclear receptor including AR consists of four functional regions, N-terminal regulatory domain (NTD), a DNA binding domain (DBD), hinge region (H), ligand binding domain (LBD) and C-terminal (C) (Figure 1). The DBD and LBDs of progesterone receptor (PR), estrogen receptor (ER), mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are structurally similar in many ways to AR, but these steroid receptors have certain functional specificity (Li & Al-Azzawi 2009; Rang et al. 2011).

Androgen receptor contains eight exons (A, B, C, D, E, F, G, and H). Exon A is responsible for transactivation and covers around 50% of the coding region, exons B and C are translated to binding domain of DNA and exons D-H to binding domain of hormones (Taplin 2007).

Figure 1: Androgen receptor functional regions (NTD: N-terminal regulatory domain, DBD: DNA binding domain, H: hinge region, LBD: Ligand binding domain, C: C-terminal

NTD domain of AR consists of half coding sequence of AR and provides a place for the majority of AR transcriptional machinery and functioning of coregulators and acts as the primary mediator for androgen effects. NTD structure alters when it interacts with other proteins with DNA (Chmelar et al. 2007).

DNA binding domain (DBD) have two zinc finger domains and C terminal extension (Mader et al. 1993). First zinc finger acts as a mediator for DNA identification through the interaction between certain base pairs so allowing the receptor to bind itself with DNA (Hard et al. 1990). Second zinc finger provides stability to this DNA receptor complex (Schoenmakers et al. 1999).

Hinge region is present between the DBD and LBD and plays an important role in commanding the transactivation potential of the androgen receptor and also provides important sites for acetylation, phosphorylation and degradation (Zhou et al. 1995; Tanner et al. 2004).

The ligand binding domain (LBD) facilitates and act as a mediator for the binding of androgenic ligands to AR. Ligands cause structural change in the LBD and bending of helix 12 back into the ligand-binding space (Li & Al-Azzawi 2009). Androgen receptor (AR) function is dependent on the co-operation between N-terminal and ligand binding site (Ikonen et al. 1997).

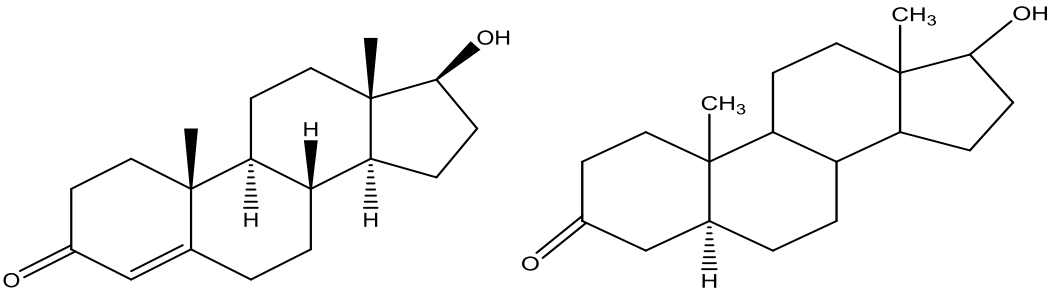
C – Terminal domain is specific according to the class of nuclear receptor and ligand binding module is present on this domain (Rang et al. 2011).

2.1.2 Physiological functions

AR is expressed in various tissues including prostate, liver, central nervous system (CNS) and skeletal muscles. Prostate, adrenal gland and epididymis have highest levels of AR. Two types of effects are caused by the activation of AR, androgenic effects and anabolic effects. Effects that are associated with the reproductive tissues e.g. testis and seminal vesicles are known as androgenic effects whereas effects of androgen in muscles and bones are known as anabolic effects. Male sexual characteristics like hair growth, maintaining libido, sperm production, muscles strength, and erythropoiesis are developed and maintained by functional AR (Gao et al. 2005).

Androgens exert their effects by acting on AR and are needed for the normal growth and functions of prostate gland (Roy et al. 1999). Testosterone is synthesized mostly in testis, then it is transported to target tissues where it is metabolized to active metabolite DHT by an enzyme 5α-reductase (Figure 2 and 3).Testosterone is responsible for various effects in human body tissues. These different kinds of effects depend on the metabolism of testosterone to two active steroidal compounds, dihydrotestosterone (DHT) and estradiol. There are 2 forms of 5α-reductase: Type I and Type II. Type I is mostly present in skin, liver and bones whereas Type II is found in genitals in men and women. Dihydrotestosterone has stronger affinity to bind with androgen receptor and

activation of genetic expression (Brunton 2006). Binding of testosterone and DHT to androgen receptor produce biological effects and also induce AR transcriptional activity. This transcriptional activity is dependent on the coregulators and their interaction with androgen receptor (AR) and phosphorylation of AR and AR coregulators (Heinlein & Chang 2004).



Testosterone Dihydrotestosterone (DHT)

Figure 2: Structures of testosterone and dihydrotestosterone (DHT)

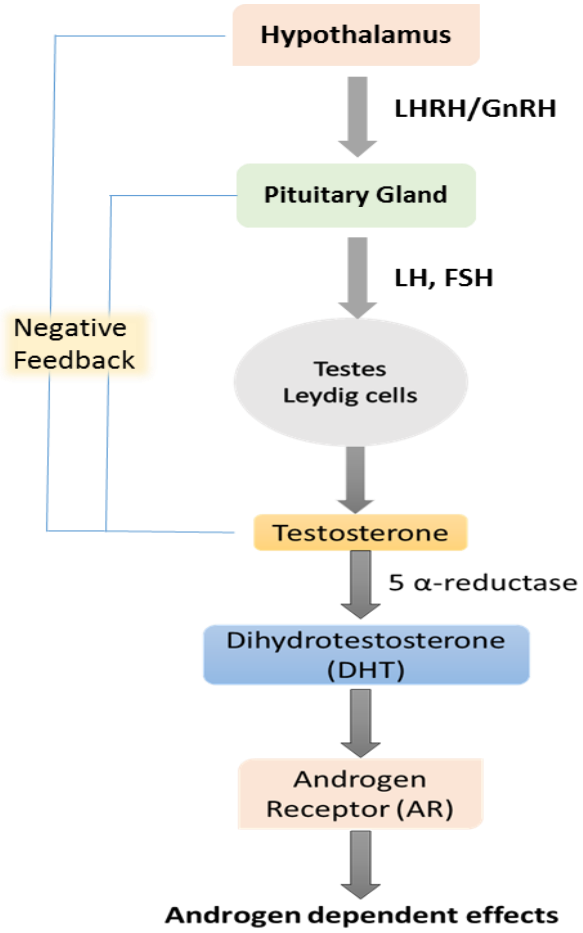


Figure 3: Mechanism to produce androgen dependent effects

2.1.3 AR interacting proteins

AR function is regulated by the interaction with the several nuclear proteins. These proteins are divided into three classes: Components of general transcriptional machinery, coregulators (coactivators or corepressors) and specific transcription factors (Heemers & Tindall 2007).

Coregulators acts as signaling linkage in receptors and transcriptional machinery. Coregulators are divided into co-activators, co-repressors, co-integrators and median protein complexes. Examples of co-activators are SRC-1, SRC-2 and SRC-3, PCAF, PBP, ARA70, L7/SPA, Trip-1, E6-AP, PGC-1 and SRA. Examples of co-repressors are NCoR, SMRT, SUNCoR and NURD. Examples of co-integrators and median protein complexes are CBP/p300 and DRIP/TRAP/SMCC respectively (Janne et al. 2000).

2.1.4 Role of AR in diseases

There are various diseases which are associated with the mutations in AR for example androgen insensitivity syndrome (AIS), infertility, prostate cancer and Kennedy's disease (Figure 4).

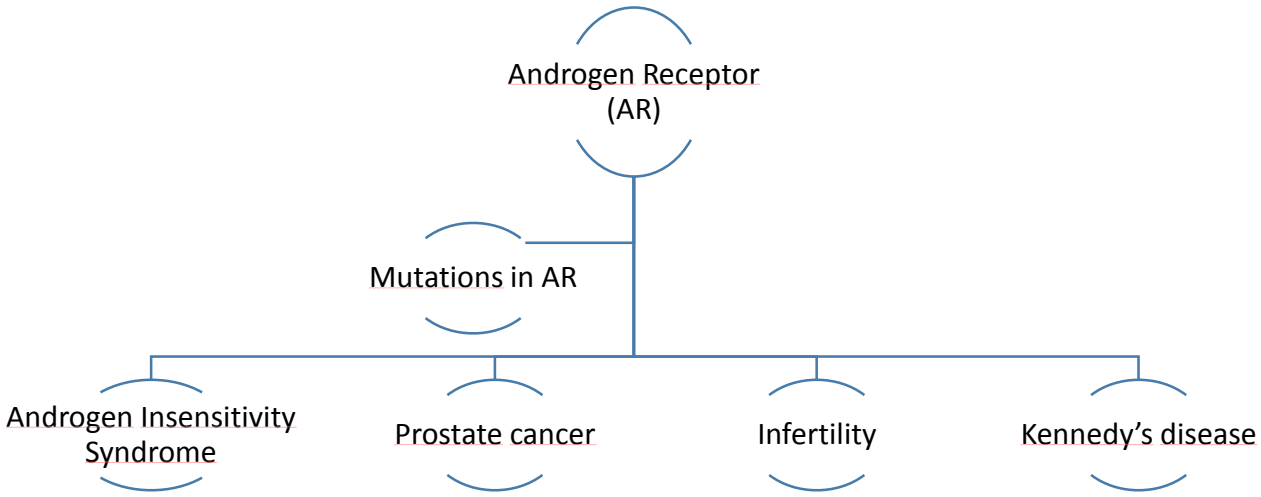


Figure 4: AR associated diseases

Androgen Insensitivity syndrome (AIS)

Mutations in the AR gene in individuals having 46, XY karyotype are believed to develop end-organ resistance to androgens (testosterone and dihydrotestosterone) called as androgen insensitivity syndrome (AIS) (Chang et al. 1995; Lee & Chang 2003). Persons with AIS have following peculiar features: external female genitalia, short blind ending vagina, absence of epididymides, vas deferens and seminal vesicles, absence of prostate, gynecomastia, no hairs on pubic and axillary regions. Levels of testosterone and lutenizing hormone rise in AIS showing resistance of androgen at hypothalamic-pituitary axis. Rise in testosterone levels also causes elevated levels of estrogens, resulted in development of more female features in AIS persons (Quigley et al 1995; Brinkmann 2001).

Kennedy's disease

Kennedy's disease is also called X-linked spinal and bulbar muscular atrophy. Kennedy's disease is a rare, incurable X-linked genetic progressive neuro-muscular degenerative disease. Degeneration of motor neurons is caused by the elevated number of CAG repeats encoding a polyglutamine stretch inside AR. Symptoms of this disease are proximal and bulbar weakness, muscular atrophy, fasciculations, gynecomastia and postural tremors (Ellerby et al. 1999; Sperfeld et al. 2002).

2.2 Androgen receptor and prostate cancer

According to National Cancer Institute USA, "Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems". Prostate cancer is one of the most prominent and lethal cancers in the world. Prostate cancer is the second most common cancer worldwide for males and the fifth most common overall (Ferlay et al. 2008).

Prostate cancer is one of the most prevalent cancers in the United States and Europe. Diet, life style, genes and androgens are the major risk factors for prostate cancer (Nelson et al. 2003). Diet consists of meat, high animal fat, less vegetables and fruits are one of the culprits for the promotion of prostate cancer. Vegetables enriched with vitamin E and other antioxidants provide protection against prostate cancer (Chan & Giovannucci 2001).

Standard treatment of prostate cancer involves regular monitoring of cancer patients, surgical removal of prostate gland, radiation therapy involving external beam radiation, brachytherapy and alpha emitter radiation therapy, hormonal therapy, immune therapy or biologic therapy, and bisphosphonate therapy to reduce bone pain associated with prostate cancer (Figure 5.) (Prostate cancer treatment 2014).

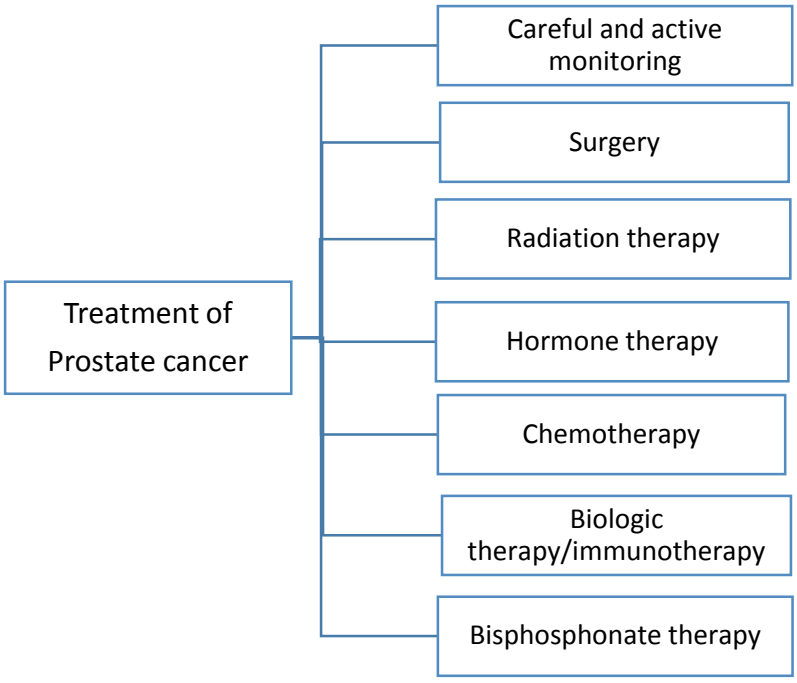


Figure 5: Standard treatment of prostate cancer (Prostate cancer treatment 2014).

Prostate cancer growth and spread is dependent on androgens, which are one of the main regulators of these cellular proliferations (Agus et al. 1999). The prostate cancer can be treated by suppression of androgen receptors (AR) and administration of anti-androgens (Figure 6.) (Hellerstedt & Pienta 2002). So treatment for prostate cancer is relied on the methods involving the reduction in the circulating androgens and androgen controlled signaling by activating androgen receptor (AR). Methods involve the castration i.e. surgical and chemical castration. Surgical castration includes getting rid of testicles whereas chemical castration includes the treatment of prostate cancer by the administration of anti-androgens or androgen deprivation therapy (ADT) (Massard & Fizazi, 2011). These castration treatments can cause resistance and ultimately resulted into the increase in the cancer tumor cells, prostate specific antigen (PSA) and

androgens. This dangerous state of prostate cancer is called as 'castration resistant prostate cancer (CRPC). Other treatment options are radiation and chemotherapy but these treatment options can't be used alone. Androgen receptor antagonists and hormonal therapies are the main options for the treatment of prostate cancer (Ahmed et al. 2014).

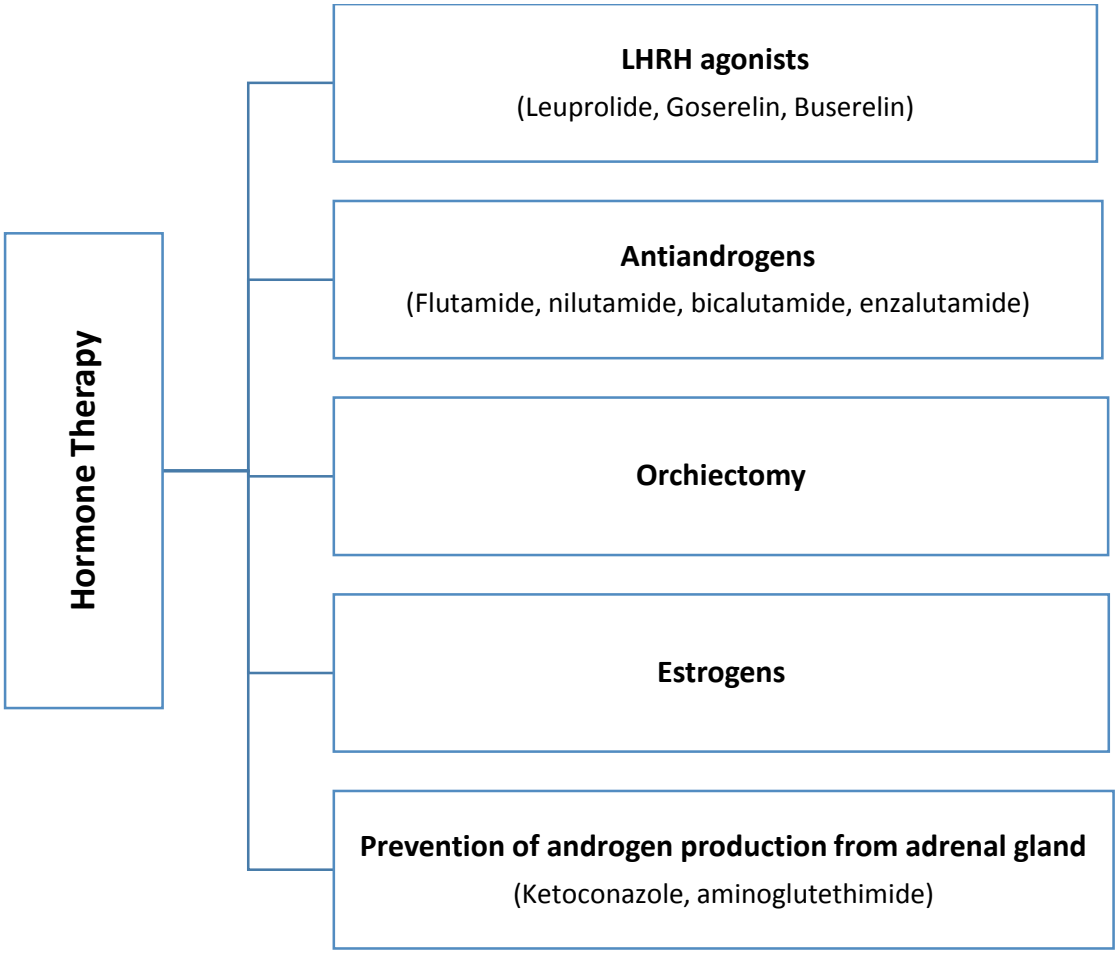


Figure 6: Hormone therapy for prostate cancer (Prostate cancer treatment 2014).

Prostate specific antigen (PSA) is the most sensitive biomarker for the diagnosis of prostate cancer and to check and evaluate the therapy's response. PSA is a glycoprotein, belongs to the kallikrein family of serum proteases. In normal conditions, PSA is released into the glandular ducts and causes the degeneration of high molecular weight proteins to stop the coagulation of the semen. During the proliferation of prostate cancer, abnormalities develop in the prostate ducts which causes the increased levels of PSA in extracellular space and ultimately into the blood circulation.

Diethylstilbestrol (DES)

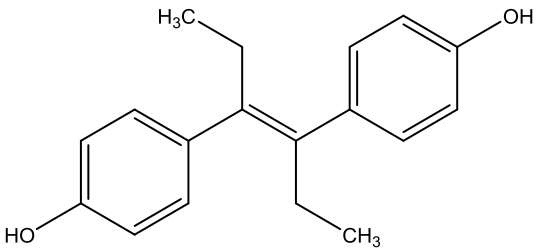


Figure 7 : Structure of Diethylstilbestrol

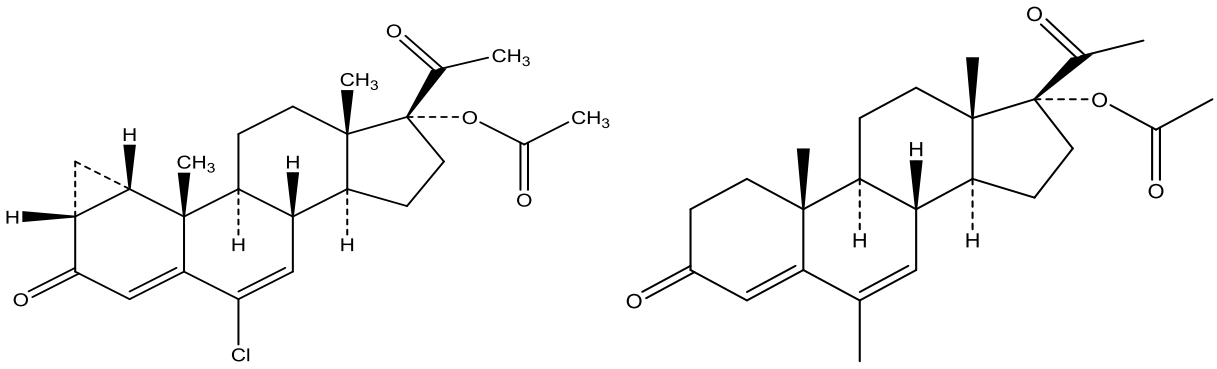
Diethylstilbestrol is the oldest compound used for the treatment of prostate cancer (Figure 7). Mechanism of action of DES is to suppress the secretion of pituitary luteinizing hormone (LH) by causing the negative feedback effect on hypothalamus and pituitary gland. Estrogens increase the levels of sex hormone binding and prolactin secretion by pituitary gland. Estrogens decrease the testosterone production in the testes. VACURG trials initiated in 1960's provided the data about the cardio-toxicity associated with the DES usage in 3 mg/day dose. DES associated cardiovascular side effects are decrease in antithrombin III, increase in production of coagulation factors I, II, VII, IX and X, hypofibrinolysis, and decreased plasminogen activator (Malkowickz 2001).

2.3 Androgen receptor antagonists

Androgen receptor antagonists (antiandrogens) are used for the treatment of prostate cancer; these drugs inhibit the binding of androgens to the androgen receptor or inhibit 5α-reductase. Anti-androgens can be classified into two main groups: steroidal and non-steroidal antiandrogens. The use of antiandrogens as monotherapy can be beneficial not only for the treatment of prostate cancer but to preserve function of gonads and improving the quality of reproductive life and bone demineralization (Miyamoto et al. 2004).

2.3.1 Steroidal antiandrogens

Examples of steroidal antiandrogens are progesterone analogues cyproterone acetate, and megestrol acetate (Figure 8). Steroidal antiandrogens work as antagonist at androgen receptor and agonist at progesterone receptors. Steroidal antiandrogens are not used now because of their potential side effects and low effectiveness (Miyamoto et al. 2004).



Cyproterone acetate Megestrol acetate

Figure 8: Structures of steroidal anti-androgens

Cyproterone acetate (CPA)

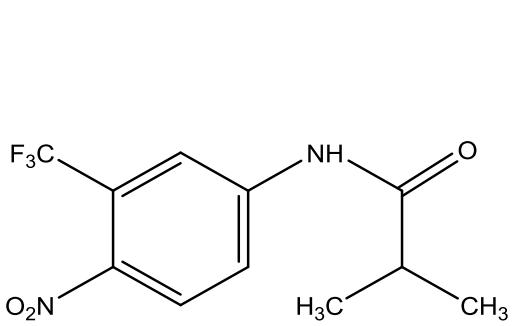
Cyproterone acetate (CPA) is a steroidal progesterone analogue. It is used for the treatment of prostate cancer because of its anti-androgenic properties. CPA is also used for the treatment of other diseases like acne, female hirsutism, and breast cancer in females, hyper sexuality and sexual deviation in males. CPA exerts its mechanism of action by inhibiting the peripheral actions of testosterone and suppressing the gonadotropin release through negative feedback on pituitary gland (Savidou et al 2006). Cyproterone acetate is administered at a dose of 100 mg twice daily 5-7 days before LHRH analogue and used for 3-4 weeks (Mahler et al. 1998). CPA is believed to have good tolerability but cases of hepatic failure reported following its usage. The mechanism behind this hepatotoxicity is not well characterized. Hepatotoxicity decreases the overall survival period of prostate cancer patients (Kim et al. 2014). CPA causes various complications including cardiovascular complications, loss of libido, erectile dysfunction, gynecomastia and central nervous system effects (Miyamoto et al 2004).

Megestrol acetate

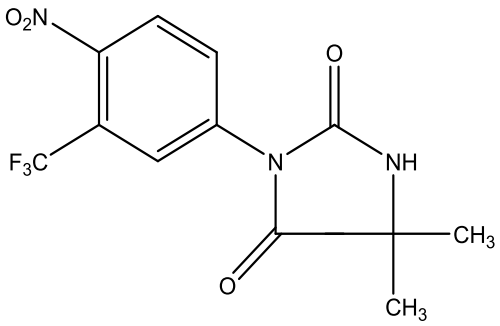
Progesterone derivative megestrol acetate is potent inhibitor of 5α-reductase so causing the inhibition of conversion of testosterone to its active metabolite i.e. dihydrotestosterone (DHT). It is used for the treatment of prostate cancer in men and effective dose is 8-10 mg daily along with 1-2 mg/day of testosterone. Various studies have shown that the use of progesterone as treatment for prostate cancer and breast cancer caused significant decrease in elevated prostate-specific antigen (PSA) (Kaore et al. 2012).

2.3.2 Non-steroidal anti-androgens

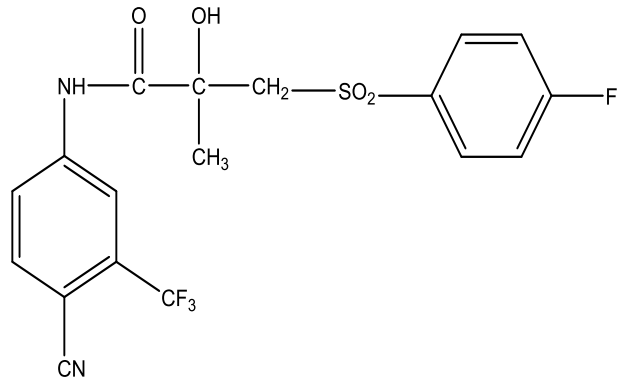
In order to counter the adverse effects associated with the use of steroidal anti-androgen, non-steroidal anti-androgens were developed. Examples of non-steroidal anti-androgens are nilutamide, flutamide, and bicalutamide (Figure 9.) (Chen 2009). Non-steroidal antiandrogens stop the binding of testosterone and Dihydrotestosterone (DHT) to androgen receptors (AR) (Hellerstedt & Pienta 2002). Non-steroidal antiandrogens are chemical derivatives of anilides. These agents stop binding of androgens i.e. testosterone and dihydrotestosterone to AR in CNS, which interferes the negative feedback of testosterone on gonadotropin release. Steroidal antiandrogens cause the decrease in the levels of testosterone whereas non-steroidal antiandrogens increase testosterone levels so causing less impairment in sexual desire and potency (Reid et al. 1999). Ezalutamide has been approved by FDA in August 2012 for the treatment of castration resistant prostate cancer (Ning et al. 2013).



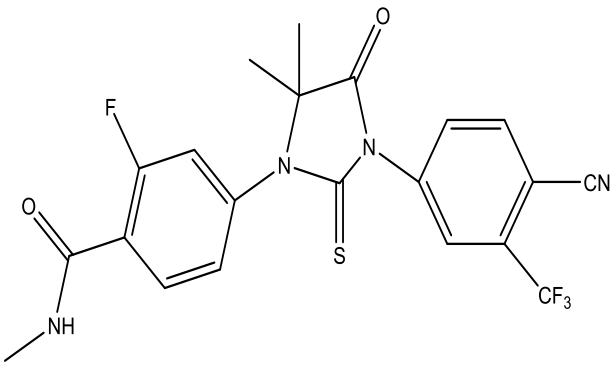
Flutamide



Nilutamide



Bicalutamide



Enzalutamide

Figure 9: Structures of non-steroidal anti-androgens

Flutamide

FDA approved flutamide in 1989 and it is normally combined with LHRH agonists for the treatment of advanced and metastatic prostate cancer (Helsen et al. 2014). Flutamide absorption from gastrointestinal tract is rapid. After metabolism, flutamide is converted to 2-hydroxyflutamide (active compound). Excretion is through renal route and half-life is around 5-6 hours. Flutamide is administered at a dose of 250 mg, 3 times a day. Flutamide exerts its action by preventing the binding of DHT to its receptor and also causes the inhibition of translocation of DHT already attached to the receptor inside nuclei. Common side effects associated with the use of this drug are gynecomastia, breast pain, hot flashes, diarrhea, and liver disturbances (Mahler et al. 1998). Flutamide is usually administered with LHRH agonists and not administered as monotherapy to treat metastatic prostate cancer because EORTC trial explained that flutamide causes sexual infertility in men and has poor efficacy (Helsen et al. 2014).

Nilutamide

Nilutamide is an anti-androgenic drug that blocks the action of testosterone and dihydrotestosterone (DHT). Nilutamide can be considered as pure anti androgen because it shows negligible affinity for progesterone, estrogen, or glucocorticoid receptors. Nilutamide is usually administered in combination therapy with LHRH agonists in castrated patients. Nilutamide improves the quality of life in castrated patients (Harris et al. 1993). Nilutamide is almost completely absorbed through gastrointenstinal tract. It takes 2.8 hours to reach peak plasma concentration. Metabolism is extensive and nilutamide has long half-life (56 hours) which ensures its once daily dose. Nilutamide is usually administered at a dose of 300 mg single oral dose daily for one month and then 150 mg daily is administered for the treatment of metastatic prostate cancer. Nausea, vomiting, increase in hepatic transaminases, alcohol intolerance, hot flashes, gynecomastia and delayed adaptation to darkness are the drug associated adverse effects (Mahler et al. 1998). One 8.5 years follow up study has shown that nilutamide when combined with orchiectomy in advanced prostate cancer patients lead to the better survival and improved the quality of life relieving bone pain of these patients compared to orchiectomy and placebo (Dijkman et al. 1997).

Bicalutamide

FDA approved this drug for the treatment of prostate cancer in 1995. Bicalutamide is the most common non-steroidal anti-androgen, currently used in the treatment of prostate cancer. Bicalutamide has better efficacy, longer elimination half-life and fewer side effects as compared to flutamide and nilutamide; it also has stronger connection with androgen receptor as compared to other first generation anti-androgens. Its half-life is almost one week and ensures its once daily dosage (Goa & Spencer 1998). Bicalutamide has good bioavailability and absorption through oral route. This drug has two types of enantiomers R and S. R enantiomer has anti-androgenic activity and elimination half-life of 7 days (Mahler et al. 1998). Approved daily doses of bicalutamide in complete androgen blockade is 50 mg and 150 mg is used as monotherapy. Molecular dynamics-based simulations explained that attachment of bicalutamide to ligand binding part of AR causes the dislocation of helix 12 and deformity of the coactivator part which leads to formation of inactive complex (Osguthorpe & Hagler 2011; Helsen et al. 2014). Gynecomastia, hot flashes, liver disturbances, impotence, high blood pressure and muscle weakness are common side effects associated with the use of bicalutamide (Mahler et al. 2014).

Enzalutamide (MDV3100)

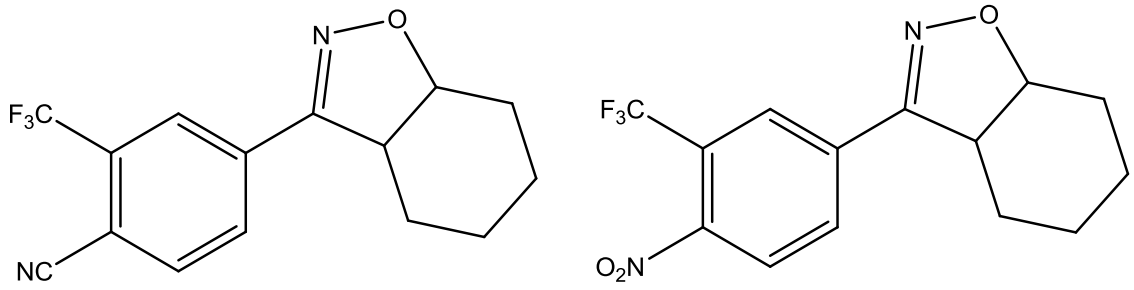
The drawbacks associated with the usage of first generation anti androgens include the development of resistance in advanced stage prostate cancer patients that resulted in the preclinical development of second generation antiandrogens. FDA approved enzalutamide in August 2012. It is different from first generation anti androgens because of its action on AR nuclear translocation, DNA binding and co-activator recruitment. This drugs binds strongly to the ligand binding domain of AR. Enzulatamide binds strongly to AR than bicalutamide but also causes the squeezing of tumor in xenograft models whereas bicalutamide only stopped tumor growth. Enzalutamide exhibited the decrease in PSA levels of more than 50% in 43-56% of patients according to different phase I and II trials (Wong et al. 2014). One international randomized phase 3 clinical trial showed that enzalutamide extended the survival of metastatic castration resistant prostate cancer patients by median of 4.8 months and subsided the death risk by 37% versus placebo (Scher et al. 2012).

2.3.3 Novel non-steroidal androgen receptor antagonists FL 442 AB and FL 425 AB

First generation of non-steroidal anti-androgens during the treatment of prostate cancer drop their potential to inhibit the androgen receptor (AR) and cancer is converted into 'castration resistant' so there is a dire need for novel androgen receptor antagonists. FL 442 AB (4-(3a, 4, 5, 6, 7, 7a-hexahydro-benzo[*d*]isoxazol-3-yl)-2-(trifluoromethyl) benzonitrile) and FL425 AB (3-(4-nitro-3-(trifluoromethyl) phenyl)-3a, 4, 5, 6, 7, 7a-hexahydrobenzo[*d*] isoxazole) are novel cycloalkane (d) isoxazole pharmacophoric fragment containing anti-androgens (Figure 10.). These recently developed anti-androgens act on the androgen receptor (AR) as effectively as or excelling than the most common non-steroidal anti-androgenic drug bicalutamide. These androgen receptor ligands (FL 442 AB and FL 425 AB) (Figure 8) have been synthesized by PhD Juha Pulkkinen and his coworkers in School of pharmacy, University of Eastern Finland (UEF) (Poutiainen et al. 2012).

Preclinical pharmacology studies suggest that FL 442 AB action is specific to AR, expresses clear antiandrogenic activity and in prostate cancer cell models it shows the expression of mutated AR ligand binding domain (LNCaP) and amplified AR (VCaP). FL 442 AB has half-life of 8 hours according to pharmacokinetic data in mice. FL 442 AB can be compared with enzalutamide because it is equally efficacious for stoppage of prostate cancer cell proliferation as enzalutamide. FL442 maintains its antiandrogenic potential with enzalutamide-activated ARF876L (Poutiainen et al. 2014).

There is limited information about the metabolic and inhibition potential aspects of these new ligands. Therefore it is required to get information about the metabolism and inhibition potential of these novel androgen receptor antagonists.



FL 442 AB ($C_{15}H_{13}N_2F_3O$) (295.1053)

FL 425 AB ($C_{14}H_{13}N_2F_3O_3$) (315.0951)

Figure 10: Chemical structures of novel androgen receptor antagonists

3. AIMS OF THE STUDY

The prime objective of this study was to obtain information about the metabolism of two androgen receptor ligands FL 442 AB and FL 425 AB, and to study the inhibitory effect of these antagonists on different cytochrome enzymes (CYP 1A2 and CYP 3A4)

Specific aims of this study were (Figure 11.):

i. To get the basic laboratory practice for me.
ii. To study the rate of disappearance of 1 µM FL 442 AB and FL 425 AB ligands in microsomal CYP incubation conditions.
iii. To compare the rate of disappearance of these ligands between mice and human.
iv. To generate mixture of metabolites of FL 442 AB and FL 425 AB and to study the effect of these mixtures on expression of androgen dependent genes in LNCAP and VCAP prostate cancer cell cultures.
v. To study the inhibition of hepatic coumarin 7-hydroxylation (CYP2A6), 7-Ethoxyresorufin O-deethylation (CYP 1A2), 7-benzyl-4-trifluoromethylcoumarin O-debenzylation (CYP 3A4) by FL 442 AB and FL 425 AB and determination of IC 50 values.

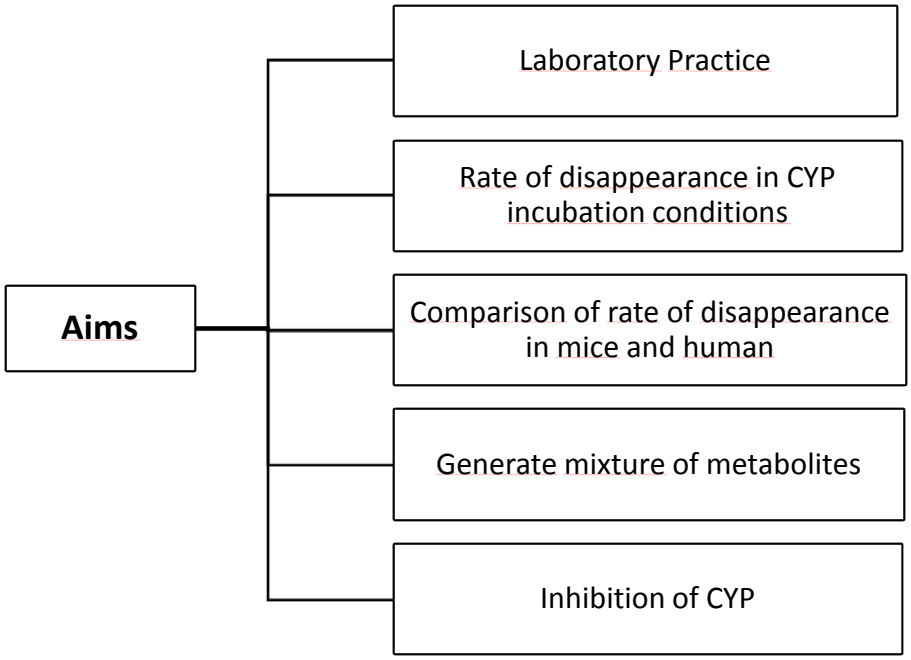


Figure 11: Aims of the study

4. METHODOLOGY

4.1 Materials

4.1.1 Chemicals

- 1 M phosphate buffer (pH 7.4)
- 1 M and 100mM Tris HCl buffer (pH 7.4) Ultrapure by MP Biomedicals France
- 30% Trichloro acetic acid (TCA) by Sigma Aldrich Germany
- 1.6 M Glycine-NaOH buffer (pH 10.4)
- NADPH regeneration system (prepared by laboratory technician)

  200 ml of NADPH regeneration system is composed of 178, 5 mg NADP, 645 mg citric acid, 75 mL 0,5 M Tris HCl + 0,15 M KCl, 25 mL 0,1 M $MgCl_2$, 25 mL 0,1 mM $MnCl_2$ in 200 mL H_2O.
- 0.1 mM and 10 mM coumarin (in ethanol)
- 1 mM and 10 mM 7-Hydroxycoumarin
- Methanol
- Ethanol (99.5%) by Altia Oy Finland
- Acetonitrile (HPLC grade)
- Dichloromethane
- 20 mM Tranylcyclopropylamine in DMSO
- 1 mM 7-ethoxyresorufin in DMSO
- 100 µM Resorufin
- 10 mM 7-benzyl 4 trifluoromethylcoumarin (BFC)
- 0.2 mM 7-Hydroxy-4-trifluoromethylcoumarin (HFC)
- Stop reagent (80% Acetonitrile/20% 0.5 M Tris HCl)
- Purified Water (H_2O) MAXIMA USF ELGA instrument at 18.2 MΩ.
- Androgen receptor antagonists FL 442 AB and FL 425 AB

4.1.2 Biological Material

Pig liver samples were obtained from untreated female pigs that were used for practicing surgical procedures at the University of Kuopio. DBA/2N/kuo mice were obtained from the National Laboratory Animal Center, Kuopio University. Liver microsomes were prepared from the animals as described previously (Lang et al., 1981). Human liver samples were obtained from patients undergoing surgery to remove hepatic tumors. Liver samples were frozen in liquid nitrogen and stored at -70^{o}C. Only tumor-free tissues were used for the experiments. Baculovirus-insect cell-expressed human CYP1A2 and CYP3A4 were purchased from BD Biosciences Discovery Lab ware (Bedford, MA, USA)

4.1.3 Instruments

- VICTOR 2^{TM} plate counter fluorescence spectrophotometer (Perkin Elmer Life Sciences, Wallac, Finland)
- Agilent 1290 series rapid resolution LC system (Agilent technologies, Waldbronn, Germany)
- Agilent 6540 Q-TOF MS (Agilent Technologies, Palo Alto, CA, USA)
- Centrifuge multifuge 3L-R

4.1.4 Ethical considerations

The Ethics Committee for Animal Experiments, University of Kuopio approved the animal tissue samples. The use of surplus human tissue of patients undergoing surgery to remove hepatic tumors was approved by the Ethics Committee of the University of Oulu.

4.2 Enzyme assays

4.2.1 Laboratory training

Training of coumarin 7-hydroxylation

Coumarin 7-hydroxylation was determined through 1 mL mixture containing 100 µL 1 M phosphate buffer pH 7.4, 10 µL 0.1 mM coumarin, 5 µL human liver microsomes and 400 µL NADPH regenerating system. This mixture was incubated at 37^{o}C and 100 µL samples stopped by 20 µL 30 % TCA at time points 5, 10, 30, 40, 50, 60, 70 and 80 minutes. Three blank samples did not contain either microsomes, coumarin or NADPH and were done otherwise similarly. 140 µL 1.6

M Glycin-NaOH buffer pH 7.4 was added to the samples and measured with Victor multiplate reader using excitation 350 nm and emission 450 nm.

Training of inhibition of coumarin 7-hydroxylation

Laboratory training about inhibition was performed by making reaction mixture and inhibition by 100 µL compound mixture each containing 5 µL 1 M Tris HCl pH 7.4, 1 µL 250 µM coumarin, 5 µL pig liver microsomes and 25 µL NADPH regenerating system. 5 µL 20% DMSO also added in reaction mixture. Tranylcyclopropylamine was used as an inhibitory compound and 5 µL serial dilutions (20, 4, 0.8, 0.16, 0.032, 0.0064, 0.00128 and 0 mM) tranylcyclopylamine were added in inhibition to the compound mixture separately. These mixtures were then pre incubated at 37^{o}C for 10 min. Reaction was stopped after 20 minutes of incubation by addition of 10 µL 30% TCA. Two blank samples were used, one sample was without microsomes and tranylcyclopropylamine and other without coumarin and DMSO. Standards of 7-hydroxy coumarin (0, 10 nM, 25 nM, 50 nM, 100 nM, 0.25 µM) 100 µL in volume were also added to multi-well plate wells. 140 µL 1.6 M Glycin-NaOH buffer pH 7.4 added to the samples and measured with Victor multiplate reader using excitation 350 nm and emission 450 nm.

4.2.2 FL 442 AB and FL 425 AB oxidative metabolism

CYP oxidative metabolism experiment was performed by making 1 mL mixture containing 100 µL 1 M phosphate buffer pH 7.4, 10 µL 0.1 mM androgen ligand FL 442 AB or FL425 AB, 5 µL microsomes, and 485 µL purified water. 400 µL NADPH was added in the last to start the reaction and then incubated at 37^{o}C. Three mouse liver (M1, M2 and M3) and two human liver (H1 and H2) microsomes were used in this experiment. 100 µL samples were taken to 300 µL acetonitrile (HPLC Grade) at time points 5 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 70 min, 80 min. Three blank 200 µL samples did not contain either microsomes, androgen ligand or NADPH respectively. After 80 minutes reaction in blank sample tubes were stopped with 600 µL acetonitrile. Samples were then centrifuged for 10 min at 10,000 RPM. 300 µL supernatant was transferred to the Eppendorf tube. Evaporated samples were dissolved in 50% methanol, then stored at -70^{o}C in refrigerator. Later these samples were analyzed (MS Analysis).

4.2.3 Production of oxidative metabolites of FL 442 AB and FL 425 AB to study their effect on AR response in-vitro

To observe the effects of the oxidative metabolites of FL 442 AB and Fl425 AB on prostate cancer cell cultures LNCAP and VCAP, 5 ml reaction mixture was prepared for both androgen ligand containing 500 µL 1 M phosphate buffer pH 7.4, 50 µL 1 mM androgen ligand, 100 µL microsomes and 2350 µL of purified water. 2000 µL NADPH was added in the last to start the reaction and then incubated at 37^{o}C for 2 hours. Three blank samples did not contain either microsomes, NADPH or androgen ligand respectively. 2.5 mL samples were transferred to 5 mL dichloromethane tubes, mixed and centrifuged for 5 min at 3000 RPM. Dichloromethane (lower phase) was transferred to another tube with the help of Pasteur pipettes. 5 mL dichloromethane was added to water phase, mixed and centrifuged again. Dichloromethane (lower phase) was transferred again to another tubes with the help of Pasteur pipettes. 0.5 mL sample was collected in Eppendorf tubes from the combined dichloromethane fraction and then evaporated. These samples were analyzed to check the level of antagonists and metabolites. In remaining samples 50 µL ethanol added, mixed and closed. Evaporated the samples and stored in refrigerator. Then analyzed the effects of these oxidative metabolites on LNCAP and VCAP prostate cancer cell cultures.

Luciferase Reporter Assay

One day before transfection, COS-1 cells (from ATCC) were seeded onto 24-well plates in 1 ml of DMEM (Dulbecco's modified Eagle medium, Gibco) containing 10% dextran-charcoal-treated fetal bovine serum (FBS) and 0.25% (vol/vol) penicillin-streptomycin (Euroclone) at a density of 70 x 10^3 cells/well. After medium change to DMEM containing 2.5% FBS, the cells were transfected for 24 h with pSG5-hAR (10 ng/well), corresponding luc reporter (100 ng/well) and internal control pCMVb (10 ng/well) by using TransIT-LT1 Transfection Reagent (Mirus Bio LCC). After the transfection (18h), the cells were exposed to vehicle (ethanol) alone, testosterone (10 nM) (100% activation). In the antagonism reporter assays, the cells were exposed simultaneously to the agonist reference compound (oxidative metabolites of FL 442 AB and FL 425 AB). After 18 h, cells were processed and luciferase activities and protein concentrations were measured and relative luciferase activities were calculated (Poutiainen et al. 2012).

4.2.4 Inhibition of hepatic coumarin 7-hydroxylation (CYP2A6) by FL 442 AB and IC 50 value determination

To see the inhibition of hepatic coumarin 7-hydroxylations by these novel AR antagonists, serial dilutions of 10 mM (3.33, 1.11, 0.37, 0.12, 0.04, 0.13 and 0mM) of FL 442 AB were prepared in ethanol. 100 µL reaction sample consisted of 5 µL 1 M tris HCl pH 7.4, 1 µL 100 µM coumarin, 5 µL 1/10 human liver microsomes, 1 µL ethanol and 63 µL purified water. Whereas 100 µL inhibition by compound sample had similar reagents as reaction sample except ethanol and in addition it contained 1 µL different concentrations of FL 442 AB. Two blank samples were used, one blank sample was without microsomes and FL 442 AB and other one was without coumarin and ethanol. This multi-well plate was kept in incubator for 10 min at 37^{o}C and reaction was started by the addition of 25 µL NADPH in all samples and kept that plate in incubator for 20 min. Reaction was stopped by the addition of 10 µL 30% TCA. Standards of 100 µL (0, 10 nM, 25 nM, 50 nM, 100 nM, 0.25 µM) of 7-Hydroxy coumarin were added to multi-well plate wells. 140 µL 1.6 M Glycin-NaOH buffer pH 7.4 was added to the samples and measured with Victor multiplate reader using excitation 350 nm and emission 450 nm.

4.2.5 Inhibition of 7-ethoxyresorufin O-deethylation (CYP1A2) by FL 442 AB and FL 425 AB

To see the inhibition of 7-ethoxyresorufin O-deethylation (CYP1A2) by FL 442 AB and FL 425 AB, serial dilutions of 10 mM (10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156 mM) FL 442 AB and FL 425 AB were prepared in ethanol. 100 µL reaction sample consisted of 10 µL 1 M tris HCl pH 7.4, 0.6 µL 1 mM 7-ethoxy resorufin, 1 µM recombinant CYP1A2 and 37.4 µL purified water. Whereas 100 µL inhibition by compound sample had similar reagents as reaction sample except 1 µL ethanol was replaced with 1 µL different concentrations of FL 442 AB and FL 425 AB. Two blank samples were used, one was without 7-ethoxy resorufin and other one was without NADPH. Reaction was started by adding 50 µL NADPH to samples. 50 µL water was added to the wells of blank 2. Multi-well plate was then incubated for 30 minutes. 100 µL 100 mM Tris HCl pH 7.4 and 0.5 µM resorufin standard were added to standard wells. Reaction was stopped with 110 µL 80% Acetonitrile/20% 0.5 M Tris HCl and measured with Victor multiplate reader using excitation 350 nm and emission 450 nm.

4.2.6 Inhibition of 7-benzyl-4-trifluoromethylcoumarin O-debenzylation (CYP3A4) by FL 442 AB and FL 425 AB

To see the inhibition of 7-benzyl 4-trifluoromethylcoumarin O-debenzylation (CYP3A4) by FL 442 AB and FL 425 AB, serial dilutions of 10 mM (10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.156mM) FL 442 AB and FL 425 AB were prepared. 100 µL reaction sample consisted of 10 µL 1 M tris HCl pH 7.4, 0.5 µL 10 mM BFC, 1 µM recombinant CYP 3A4 and 37.5 µL purified water. Whereas 100 µL inhibition by compound sample had similar reagents as reaction sample except 1 µL ethanol was replaced with 1 µL different concentrations of FL 442 AB and FL 425 AB. Two blank samples were used, one was without BFC and other one was without NADPH. Reaction was started by adding 50 µL NADPH to wells of reaction and blank 1. 50 µL water was added to the wells of blank 2. Multi-well plate was then incubated for 30 minutes. 100 µL 100 mM Tris HCl pH 7.4 and 0.5 µM HFC standards were added to standard wells. Reaction was stopped with 110 µL 80% Acetonitrile/20% 0.5 M Tris HCl and measured with Victor multiplate reader using excitation 350 nm and emission 450 nm.

4.2.7 HPLC-MS analysis

The HPLC-MS of FL 442 AB and FL 425 AB were performed using an Agilent Zorbax SB-C18 column (2.1 x 50 mm, 1.8 µm particle size) together with an Agilent Infinity in-line filter (0.2 µm). The temperature of the column oven was 50°C and the injection volume was 5 µL. Methanol was used as organic phase and aqueous phase was 0.1% formic acid. A linear gradient from 20% to 90% in 5 min was applied, followed by 2 min isocratic elution with 90% MeOH and column equilibration during 3 min, giving an injection cycle of 10 min. The eluent flow rate was 0.3 mL/min. The MS equipped with an AJS electrospray ionization (ESI) source was operated in positive ionization mode. Mass range of 100–1000 was acquired using the 2 GHz extended dynamic range mode. Capillary voltage was 3500 V and fragmentor voltage 100 V. Nitrogen was used as sheath gas and drying gas, the flow rates being 11 L/min at 350°C and 10 L/min at 325°C, respectively. The nebulizer was adjusted to 45 psi. Agilent reference mass solution (m/z 121.05087 and m/z 922.00979) was used as lock mass. Argon was used as collision gas and the collision energy was 30 V when acquiring the targeted MS/MS data. All the LC/MS (MS) data was acquired using Mass hunter (B.04.00) acquisition software.

4.2.8 Data analysis

For HPLC-MS data, Agilent Mass hunter Qualitative Analysis (B.05.00) and Metabolite ID (B.04.00) software's were used for the data processing and analysis of MS spectra.

Inhibitory concentration (IC 50) value was determined using the following equation below.

$$\mathbf{IC\ 50 = I / (V_0/V_i - 1) \quad OR \quad V_i/V_o = 1(1 + I/IC\ 50)}$$

I = Concentration of Inhibitor

$\mathbf{V_i}$ = Reaction Rate with Inhibitor

$\mathbf{V_o}$ = Reaction rate without Inhibitor

5. RESULTS

5.1 Laboratory Training

Laboratory training experiment about metabolism was designed to obtain the basic practical training for performing experiments with androgen receptor antagonists. For this purpose, two experiments were conducted with coumarin 7-hydroxylation catalyzed by pig liver microsomes. In the first experiment coumarin 7-hydroxylation was determined at time points 0-80 min. In the second experiment coumarin 7-hydroxylation was inhibited by tranylcyclopropylamine and its IC_{50} value was calculated.

In first training experiment of coumarin CYP oxidative metabolism, non-fluorescent coumarin was converted to fluorescent 7-hydroxy coumarin. Fluorescence increased linearly as the concentration of 7-hydroxy coumarin increased (Figure 12).

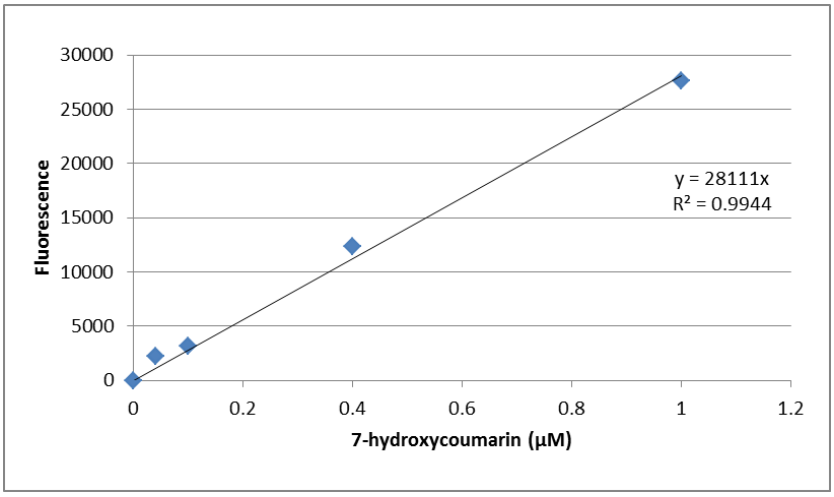


Figure 12: Standard line between concentration of 7-hydroxycoumarin and fluorescence

Amount of 7-Hydroxy coumarin increased along with the incubation time gradually from 5 minutes to 80 minutes (figure 13 A). The rate of reaction decreased gradually as coumarin converted into 7-hydroxy coumarin with the increase in time from 5 minutes to 80 minutes (Figure 13 B).

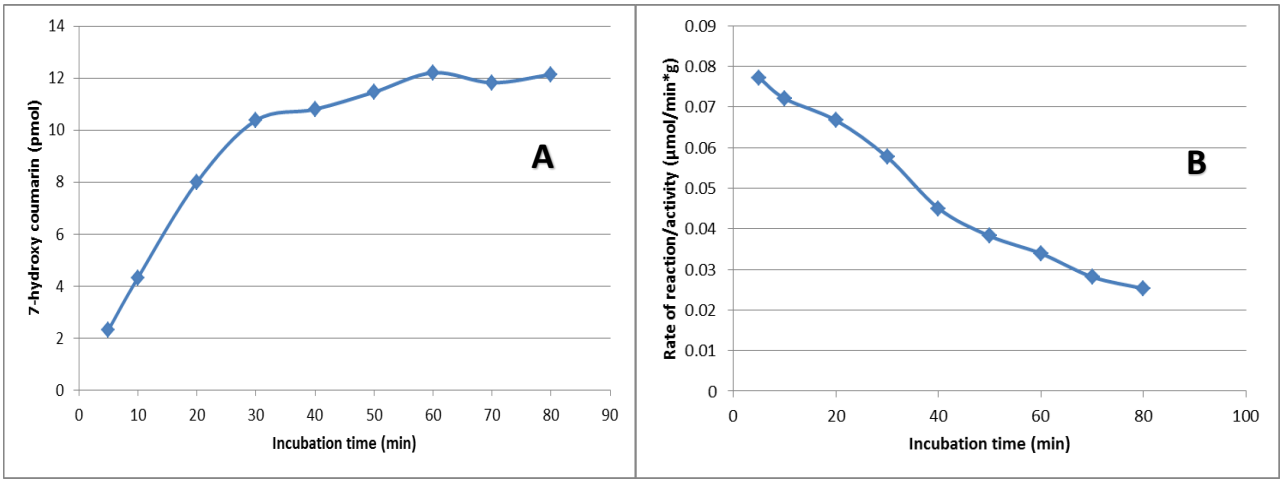


Figure 13: The effect of incubation time on coumarin 7-hydroxylation, panel A represents the formed 7-hydroxycoumarin and panel B represents the rate of coumarin 7-hydroxylation at various incubation times

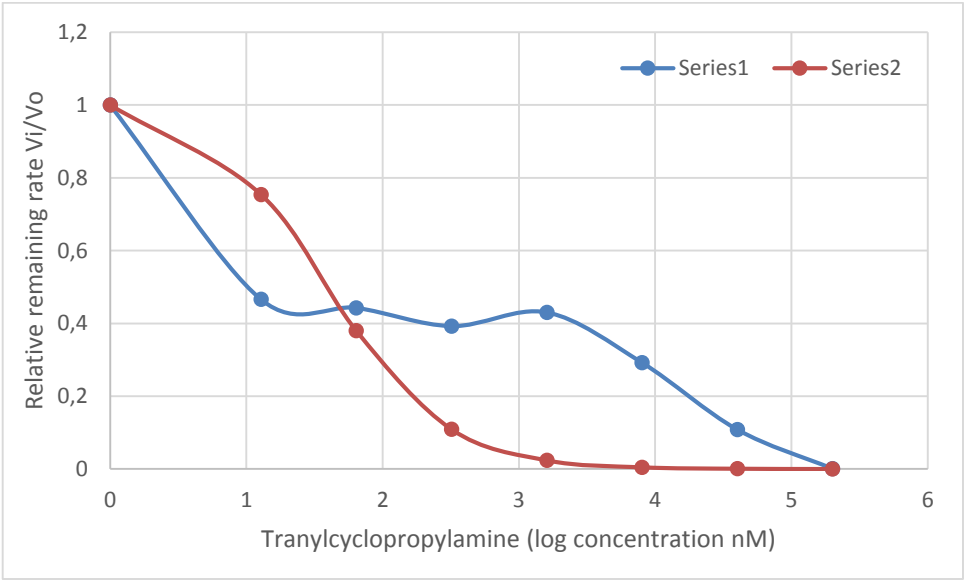


Figure 14: Inhibition of hepatic coumarin 7-hydroxylation by tranylcyclopropylamine (Blue line=Experimental, Red line=Calculated)

In the second training experiment, IC50 value of tranylcyclopropylamine for inhibition of hepatic coumarin 7-hydroxylation was 40 nM (Figure 14).

5.2 Stability and in vitro metabolites of FL 442 AB and FL 425 AB

Incubations of FL 442 AB and FL 425 AB were made with two types of microsomes i.e. mouse and human liver microsomes, reaction was started by the addition of NADPH regeneration system and then LC-MS was used to check the rate of disappearance.

Concentration of FL 442 AB was decreased to less than 50% in three types of mouse liver microsomes whereas concentration of FL 425 AB was decreased to less than 20% in mouse and less than 40% in two types of human liver microsomes (Figure 15 and 16).

Half-life of FL 442 AB in CYP incubation conditions with three types of mouse liver microsomes was 67, 60 and 64 minutes respectively. Whereas half-life of FL 425 AB in CYP incubation conditions with one mouse and two human liver microsomes was 22, 21 and 43 minutes respectively.

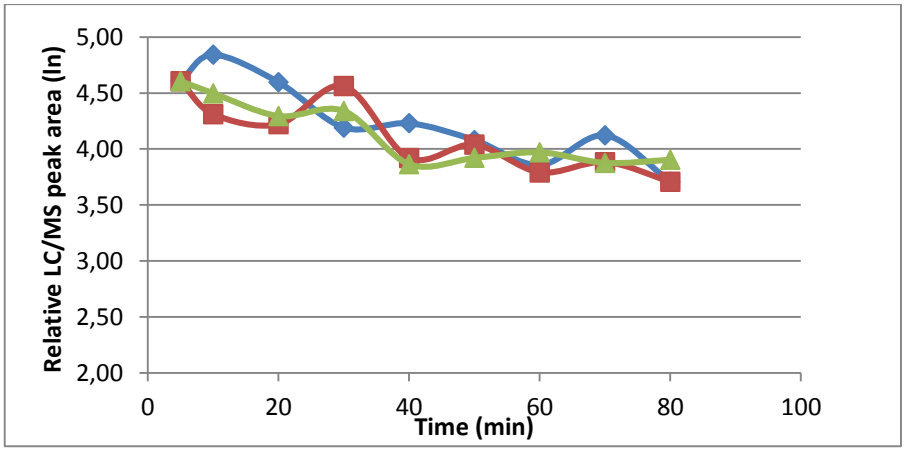


Figure 15: Rate of disappearance of 1 µM FL 442 AB in CYP incubation conditions with mouse (M) liver microsomes (Blue: M1, Red: M2, Green: M3)

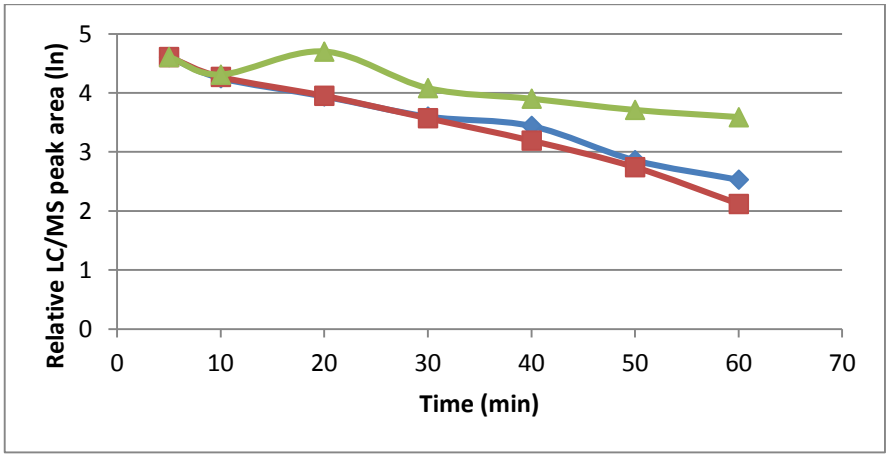


Figure 16: Rate of disappearance of 1 µM FL 425 AB in CYP incubation conditions with human (H)/mouse (M) liver microsomes (Blue: M, Red: H1, Green: H2)

5.3 FL 442 and FL 425 oxidative metabolites

To generate mixture of metabolites of FL 442 AB and FL 425 AB, human liver microsomes were used and starting concentration was 100 µM. It was observed that eight hydroxylation metabolites of FL 442 AB were formed. Metabolite 1 and metabolite 2 (RT=6,602 min and 6,606) were main metabolites of FL 442 AB and were covering 27.08% and 20.04% LC/MS peak areas respectively. Whereas all other metabolites were covering less than 15% of LC/MS peak areas (Table 1) (Figure 17).

Table 1: Retention times and accurate masses for the detected in vitro metabolites of FL 442 AB

	Compound FL 442 AB	RT(min)	Accurate mass	Exact mass	DmD	LC/MS Peak area %
1	Hydroxylation (+O)	6,602	311,0999	311,1002	– 0,3	27,08
2	Hydroxylation (+O)	6,606	311,1004	311,1002	0,2	20,04
3	Hydroxylation (+O)	4,773	311,1011	311,1002	0,9	10,88
4	Hydroxylation (+O)	5,29	311,0994	311,1002	– 0,8	5,68
5	Hydroxylation (+O)	5,583	311,1	311,1002	– 0,2	7,45
6	Hydroxylation (+O)	4,809	311,1002	311,1002	0	13,38
7	Hydroxylation (+O)	5,295	311,1006	311,1002	0,4	8,02
8	Hydroxylation (+O)	5,589	311,1004	311,1002	0,2	7,43

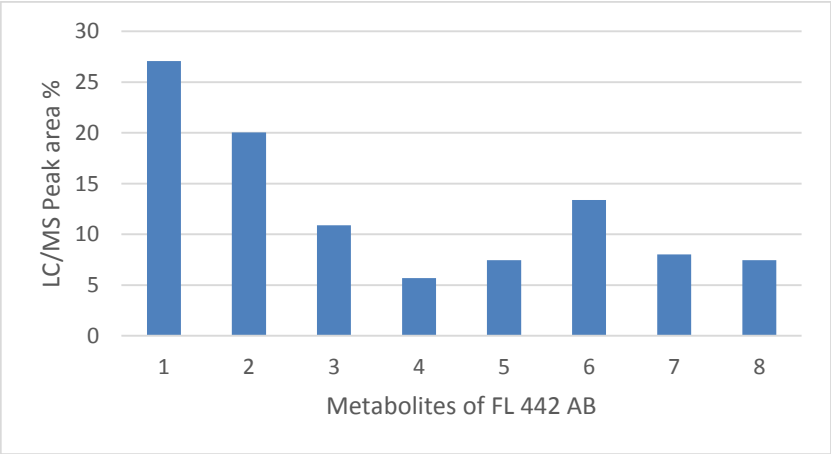


Figure 17: In vitro metabolites of FL 442 AB and their peak areas

Six hydroxylation metabolites of FL 425 AB were formed. Metabolite 5 and 6 (RT=7,343 min and 7,331) were main metabolites of FL 425 AB and were covering 23.30% and 26.08% LC/MS peak areas respectively. Whereas all other metabolites were covering less than 17% of LC/MS peak areas (Table 2) (Figure 18).

Table 2: Retention times and accurate masses for the detected in vitro metabolites of FL 425 AB

	Compound FL 425 AB	RT (min)	Accurate mass	Exact mass	DmD	LC/MS peak area %
1	Hydroxylation (+O)	5,561	331,0912	331,09	– 1,2	9,35
2	Hydroxylation (+O)	5,559	331,0905	331,09	– 0,5	12,14
3	Hydroxylation (+O)	6,361	331,0899	331,09	0,1	12,93
4	Hydroxylation (+O)	6,359	331,0895	331,09	0,5	16,18
5	Hydroxylation (+O)	7,343	331,0905	331,09	– 0,5	23,30
6	Hydroxylation (+O)	7,331	331,0906	331,09	– 0,6	26,08

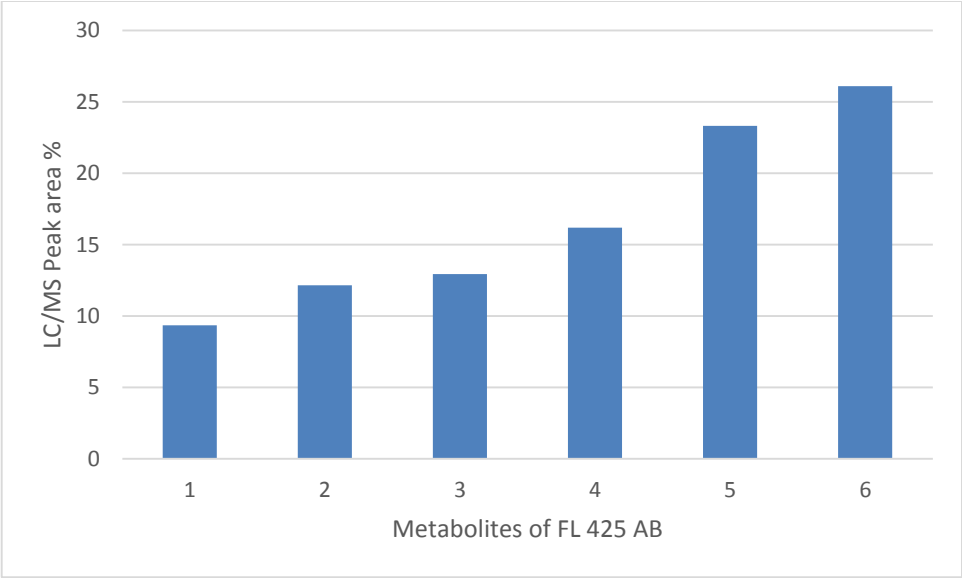


Figure 18: In vitro metabolites of FL 425 AB and their peak areas

5.4 The effect of metabolites on AR response in vitro

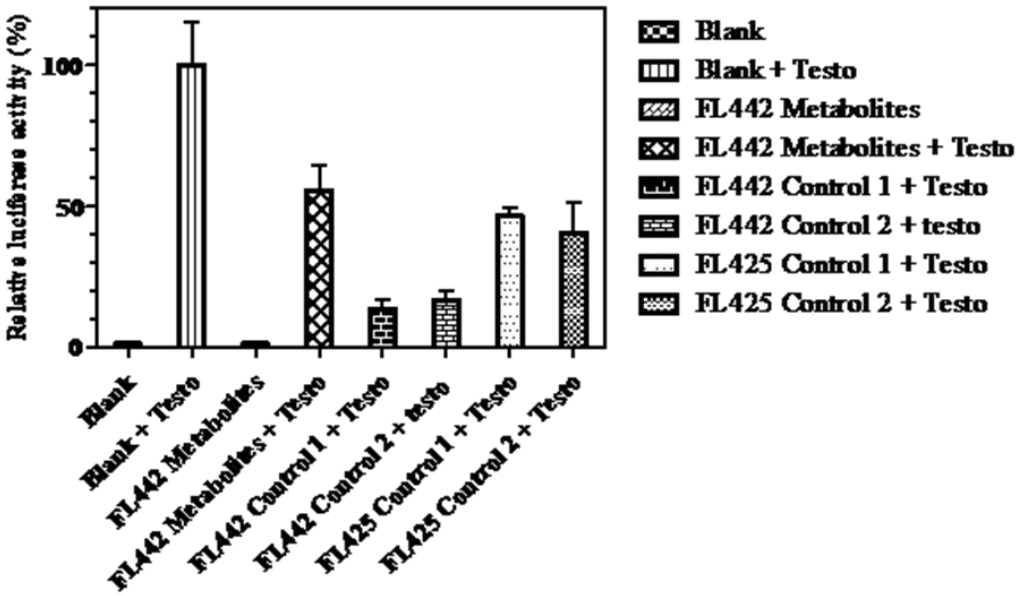


Figure 19: The effects of oxidative metabolites of FL 442 AB and FL 425 AB on AR response in-vitro

FL442 AB oxidative metabolites inhibited the transcription of androgen receptor whereas when performed same analysis on FL 425 AB metabolites it was observed that cells died and the protein content of the reaction wells was less than half of the controls and cells were visibly loose and weak so metabolites were toxic.

5.5 Inhibition of CYP enzymes

FL 442 AB inhibited weakly CYP2A6 and CYP1A2 whereas no inhibition of CYP3A4 was observed (Figures 20, 21, 22). IC-50 values are shown in table 3.

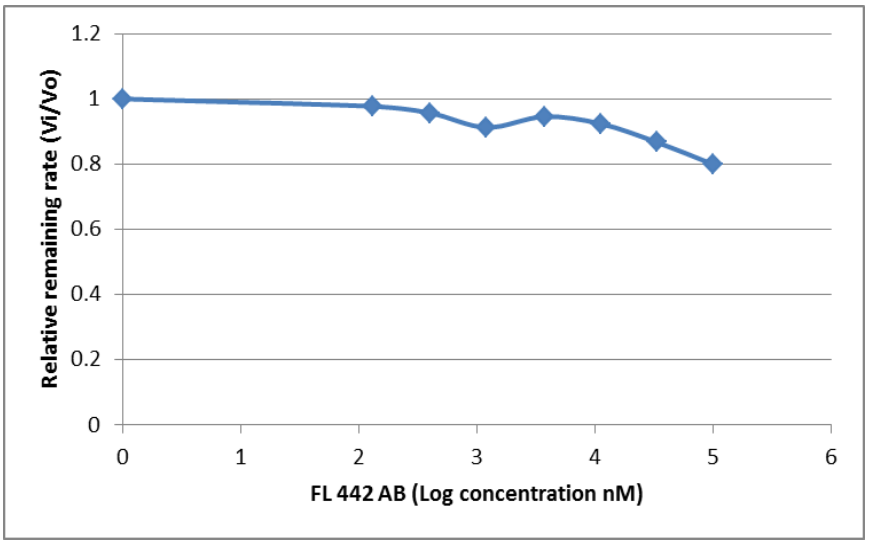


Figure 20: Inhibition of hepatic coumarin 7-hydroxylation (CYP2A6) by FL 442 AB

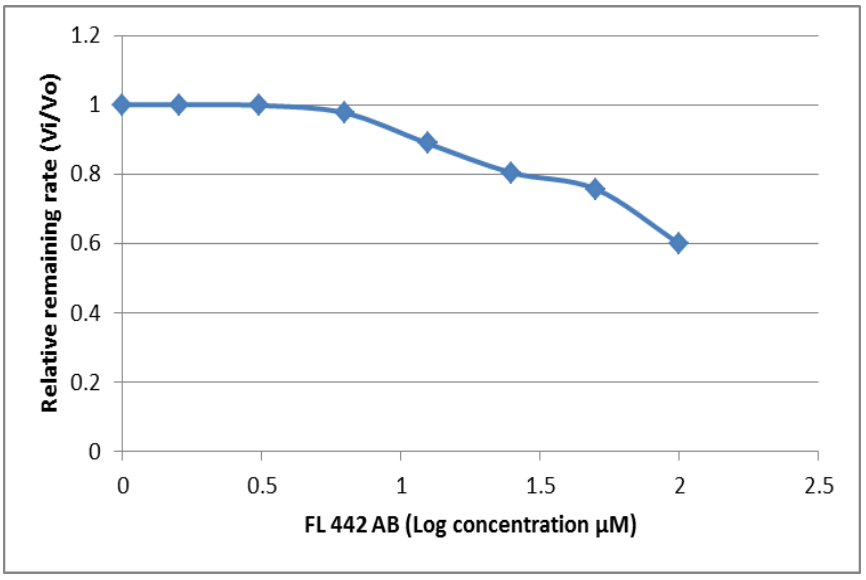


Figure 21: Inhibition of 7-ethoxyresorufin O-deethylation (CYP1A2) by FL 442 AB

7-Benzyl 4-trifluoromethylcoumarin O-debenzylation was inhibited below 0.5 µM FL 442 AB and then stimulated 2.5 times at 0.63 mM, at higher concentrations stimulation decreased.

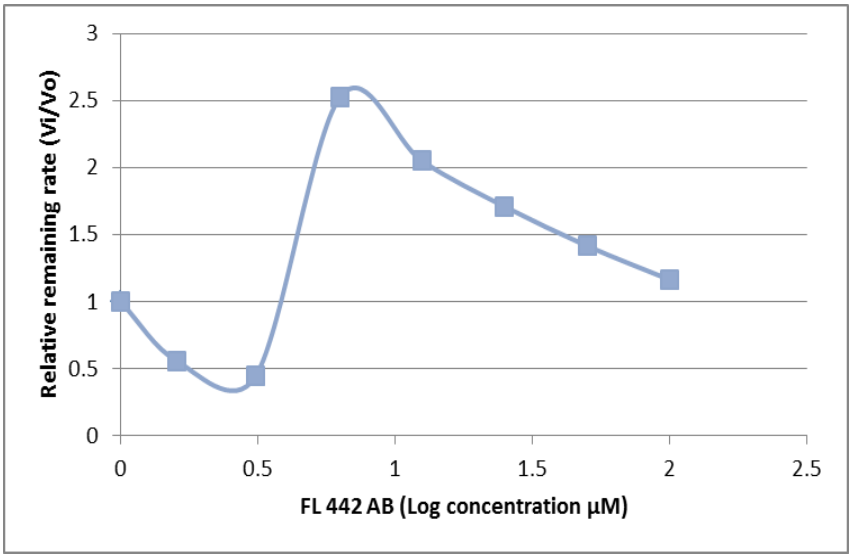


Figure 22: Inhibition of 7-benzyl 4-trifluoromethylcoumarin O-debenzylation (CYP3A4) by FL 442 AB

FL 425 AB inhibited weakly CYP1A2 whereas CYP3A4 was not inhibited (Figures 23, 24). IC 50 values are shown in table 3.

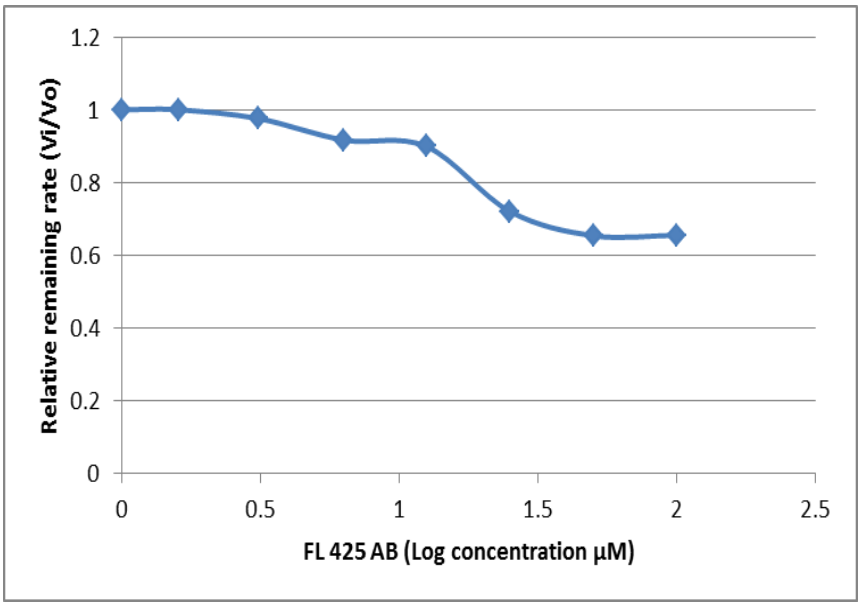


Figure 23: Inhibition of 7-ethoxyresorufin O-deethylation (CYP1A2) by FL 425 AB

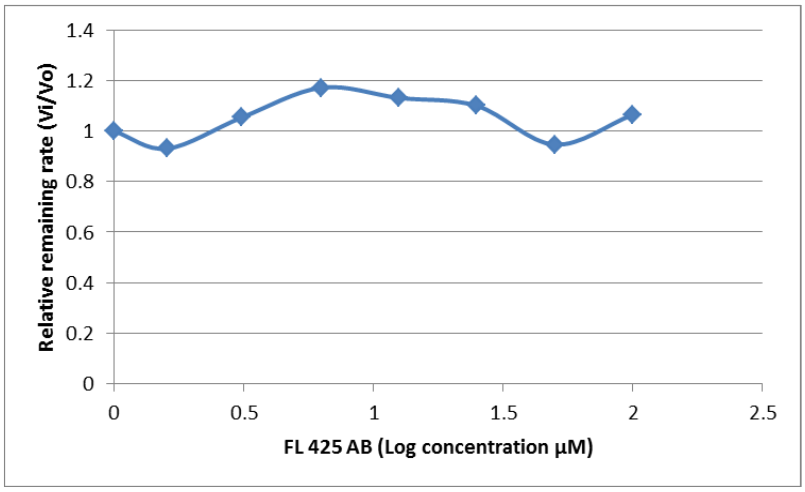


Figure 24: Inhibition of 7-benzyl 4-trifluoromethylcoumarin O-debenzylation (CYP3A4) by FL 425 AB

Table 3: IC50 of FL 442 AB and FL 425 AB

Inhibited Reaction	IC50 of FL 442 AB (µM)	IC50 of FL 425 AB (µM)
Coumarin 7-hydroxylation	330	Not performed
7-ethoxyresorufin O-deethylation (CYP1A2)	140	120
7-benzyl-4 trifluoromethylcoumarin (CYP3A4)	No inhibition	No inhibition

6. DISCUSSION

Androgen receptor (AR) is the crucial target in all forms of prostate cancer and androgen deprivation therapy (ADT) is currently the standard treatment for advanced stage prostate cancer. Use of ADT after certain period of time causes the conversion of prostate cancer to castration resistant prostate cancer. Thus, it becomes imperative to identify the underlying mechanisms that cause development of resistance. Also, discovery of new drugs like enzalutamide that can directly attach and binds to AR seem to be promising to treat castration resistant prostate cancer which is still incurable (Chen et al. 2009).

Two new non-steroidal AR antagonists FL 442 AB and its nitro analogue FL 425 AB were developed for this purpose. In the last two years, initial research studies on FL 442 AB have shown that it is selective and specific to AR and its AR binding efficiency is better than most commonly used AR antagonist bicalutamide (Gronemeyer et al. 2003; Poutiainen et al. 2012; Poutiainen et al. 2014).

Cytochrome enzymes (CYP) play important role in metabolizing lipophilic xenobiotics. It's important for the potential drugs to show metabolic stability during preclinical in vivo and in vitro testing. Therefore, this research aimed to study the rate of disappearance of 1 µM FL 442 AB and FL 425 AB ligands in microsomal CYP incubation conditions and comparison of rate of disappearance of these ligands in mice and human. Incubation of FL 442 AB and FL 425 AB were done with mouse and human liver microsomes, reaction was initiated with NADPH and then LC-MS was used to check the rate of disappearance. Concentration of FL 442 AB was decreased to less than 50% in three types of mouse liver microsomes. FL 425 AB concentration was decreased to less than 20% in mouse liver microsomes and less than 40% in two types of human liver microsomes. To generate mixture of metabolites of FL 442 AB and FL 425 AB, human liver microsomes were used and the starting concentration was 100 µM. It was observed that eight hydroxylation metabolites of FL 442 AB and six hydroxylation metabolites of FL 425 AB were formed with the addition of one oxygen molecule in parent compounds. Peaks and retention times of these metabolites were observed and analyzed by using LC-MS (Table 1 & 2). The results show that FL 442 AB and FL 425 AB have good metabolic stability. Oxidative metabolites of FL 442 AB and FL 425 AB were also investigated in-vitro for their effects on LNCAP and VCAP prostate cancer cell cultures and it was observed that these oxidative metabolites inhibited transcription of AR whereas metabolites of FL 425 AB were toxic. Three different assays were performed to study the inhibition of hepatic coumarin 7-hydroxylation (CYP2A6), 7-Ethoxyresorufin O-deethylation

(CYP1A2), 7-benzyl-4-trifluoromethylcoumarin O-debenzylation (CYP3A4) by FL 442 AB and FL 425 AB and to determine IC 50 values. CYP1A2 and CYP2A6 were inhibited weakly (IC50=330 µM and 140 µM respectively) by FL 442 AB whereas CYP1A2 was inhibited weakly (IC50=120 µM) by FL 425 AB. No inhibition of CYP 3A4 was observed by FL 425 AB. Inhibition of CYP 3A4 was observed at Initial concentrations by FL 442 AB followed by substantial stimulation of and then inhibition at higher concentrations. These results explain that novel androgen receptor antagonists FL 442 AB and FL 425 AB have shown good metabolic stability during preclinical pharmacological evaluation.

7. CONCLUSION

Metabolic aspects and inhibition potential of novel AR antagonists FL 442 AB and FL 425 AB were studied by performing different enzyme assays. Following are the major findings:

- FL 442 AB and FL 425 AB exhibited clear anti-androgenic activity.
- Eight hydroxylation metabolites of FL 442 AB and six hydroxylation metabolites of FL 425 AB were formed.
- According to in-vitro data, oxidative metabolites of FL 442 AB inhibited transcription of AR.
- According to in-vitro data, oxidative metabolites of FL 425 AB were toxic.
- Initial inhibition and then stimulation of CYP3A4 was observed at higher concentrations by FL 442 AB
- No inhibition of CYP3A4 was observed with FL 425 AB.
- CYP1A2 and CYP2A6 were inhibited weakly by FL 442 AB
- CYP1A2 was inhibited weakly by FL 425 AB

Novel AR antagonists FL 442 AB and FL 425 AB have shown good metabolic stability and these compounds have shown weak inhibition potential for CYP enzymes. However, further research studies on these compounds FL 442 AB and FL 425 AB are needful.

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