2018

# Controlling of N-Alkylpolyamine Analogue Metabolism by Selective Deuteration

Ucal, S

Portland Press Ltd.



http://dx.doi.org/10.1042/BCJ20170887

**Controlling of *N*-Alkylpolyamine Analogue Metabolism by Selective Deuteration**

Sebahat Ucal[#], Merja R. Häkkinen[#], Aino-Liisa Alanne, Leena Alhonen, Jouko Vepsäläinen, Tuomo A. Keinänen and Mervi T. Hyvönen

School of Pharmacy, University of Eastern Finland, Biocenter Kuopio, Yliopistonranta 1B, FI-70210 Kuopio, Finland.

[#] equal contribution

**Corresponding author:**
Mervi T. Hyvönen, e-mail: mervi.hyvonen@uef.fi

**Keywords:** kinetic isotope effect, metabolic switching, deuterium, flavin-dependent amine oxidoreductases, spermine oxidase, acetylpolyamine oxidase, MDL 72527, polyamines, polyamine analogues

**ABSTRACT**

Replacing protium with deuterium is an efficient method to modulate drug metabolism. *N*-alkylated polyamine analogues are polyamine antimetabolites with proven anticancer efficacy. We have characterized earlier the preferred metabolic routes of $N^1$,$N^{12}$-diethylspermine (DESpm), $N^1$-benzyl-$N^{12}$-ethylspermine (BnEtSpm) and $N^1$,$N^{12}$-dibenzylspermine (DBSpm) by human recombinant spermine oxidase (SMOX) and acetylpolyamine oxidase (APAO). Here we studied the above analogues, their variably deuterated counterparts and their metabolites as substrates and inhibitors of APAO, SMOX, semicarbazide-sensitive amine oxidase (SSAO), diamine oxidase (DAO) and monoamine oxidases. We found that targeted deuteration efficiently redirected the preferable cleavage site and suppressed reaction rate by APAO and SMOX *in vitro*. We found 3- to 6-fold decline in $V_{max}$ with moderate variable effect to $K_m$ when deuterium was located at the preferred hydrogen abstraction site of the analogue. We also found some of the metabolites to be potent inhibitors of DAO and SSAO. Surprisingly, analogue deuteration did not markedly alter the antiproliferative efficacy of the drugs in DU145 prostate cancer cells while in mouse embryonic fibroblasts, which had higher basal APAO and SMOX activities, moderate effect was observed. Interestingly, the antiproliferative efficacy of the analogues did not correlate with their ability to suppress polyamine biosynthetic enzymes, induce spermidine/spermine-$N^1$-acetyltransferase or deplete intracellular polyamine levels, but with their ability to induce SMOX.

Our data show that selective deuteration of *N*-alkyl polyamine analogues enables metabolic switching offering means for selective generation of bioactive metabolites inhibiting e.g. SSAO and DAO thus setting a novel basis for *in vivo* studies of this class of analogues.

**Abbreviations**

AdoMetDC, *S*-adenosyl-*L*-methionine decarboxylase; APAO, acetylpolyamine oxidase; DAO, diamine oxidase; DFMO, α-difluoromethylornithine; MEFs, mouse embryonic fibroblasts; MAO, monoamine oxidase; MeSpd, methylspermidine; ODC, ornithine decarboxylase; Put, putrescine; SC, semicarbazide; SMOX, spermine oxidase; Spd, spermidine; Spm, spermine; SSAO, semicarbazide-sensitive amine oxidase; SSAT, spermidine/spermine-$N^1$-acetyltransferase

## INTRODUCTION

Deuterium is a stable isotope of hydrogen containing both proton and neutron in the nucleus giving +1 mass over protium. Due to this atom mass difference protium to deuterium substitution in organic analyte molecules has been widely used to prepare internal standards for different analytes for GC-MS/MS and LC-MS/MS based quantification methods. Interestingly, deuterium-carbon bond is much stronger in comparison to protium-carbon bond and the physicochemical features of deuterium e.g. lipophilicity are different than those of protium [1]. In biological systems, deuterated molecules behave differently as compared to their non-deuterated counterparts [2]. Targeted protium to deuterium substitution in drug molecule is generally used to prevent formation of toxic or harmful metabolites, i.e. redirecting drug metabolism or to slow down the metabolism of the drug *in vivo* [3].

Mammalian polyamines spermidine (Spd) and spermine (Spm) and their diamine precursor putrescine (Put) exist at millimolar concentration in cells and they are derived from the amino acids *L*-arginine and *L*-methionine. Polyamines are positively charged under physiological conditions and thus interact with negatively charged cellular biomolecules like RNA, DNA and phospholipids [4]. Moreover, they interact with or allosterically modulate a variety of transport and receptor proteins and metabolic enzymes. Intracellular polyamine levels are strictly regulated through *de novo* synthesis, interconversion and excretion via *N*-acetylation and catabolism through terminal deaminative oxidation. Polyamines, especially Spd are essential for cell proliferation, and dysregulated polyamine metabolism is associated with neoplastic transformation of cells. Thus, polyamine *de novo* synthesis has been a target of systematic drug development for decades. Specific inhibitors for almost all known enzymes of polyamine biosynthesis pathway have been synthesized [5]. They have proved valuable research tools, but by far only alpha-difluoromethylornithine (DFMO; Elfornithine®), an irreversible binding inhibitor of ornithine decarboxylase (ODC), has been approved for clinical use as antiparasitic drug. Promisingly, it is under clinical trials as an anticancer agent as single agent or in combination with sulindac and some other anticancer or anti-inflammatory drugs [6].

Amongst the most efficient developed polyamine antimetabolites are *N*-alkylated polyamine analogues, like $N^1$,$N^{11}$-diethylnorspermine (DENSpm, *N,N'*-bis-(3-ethylaminopropyl)propane-1,4-diamine) and $N^1$,$N^{12}$-diethylspermine (DESpm, *N,N'*-bis-(3-ethylaminopropyl)butane-1,4-diamine) [6, 7]. DENSpm overcame the defects associated with the use of single biosynthesis enzyme-targeting inhibitors as it not only suppresses *de novo* polyamine synthesis but also induces catabolism and efflux of polyamines and prevents the uptake of dietary and microbiota-originated polyamines to cells and tissues. Initially, systematic studies of *N*-alkylated polyamine analogues were targeted to dissect structure-antimetabolite relationships and the most effective analogues advanced to clinical trials [8]. Metabolism and pharmacokinetic studies with *N*-diethylated analogues showed that *N*-deethylation of the analogues is the major catabolic route, and that their *in vivo* catabolism could be prevented by co-administration of $N^1$,$N^4$-bis(2,3-butadienyl)-1,4-butanediamine (MDL 72527), an inhibitor of both acetylpolyamine oxidase (APAO; official name PAOX) and spermine oxidase (SMOX), but not by semicarbazide (SC), an inhibitor semicarbazide-sensitive amine oxidase (SSAO; official name AOC3) and diamine oxidase (DAO; official name AOC1)[9]. Interestingly, APAO was shown to detoxify Spm analogue $N^1$-ethyl-$N^{11}$-[(cycloheptyl)methyl]-4,8-diazaundecane (CHENSpm) [10]. On the other hand, catabolism of dibenzylated analogue known as MDL 27659, having benzyl-3-7-3-benzyl carbon chain, yielded a monobenzylated metabolite (MDL 28560) and a completely debenzylated metabolite (MDL 26752), of which the latter metabolite was more toxic to rat hepatoma cells than the parent compound [11]. Thus,

polyamine analogue metabolism could play dual role in determining drug efficacy in connection to analogue structure and expression of APAO and SMOX in target cells.

SMOX and APAO were cloned in 2001 and 2003, respectively, which facilitated their production as recombinant proteins and more detailed analysis of degradation products and substrate properties of earlier synthesized polyamine analogues. Interestingly, while the known natural substrates of APAO and SMOX are achiral, they were found to display stereospecificity towards chiral *C*-methylated polyamine analogues [12, 13]. More importantly, the stereospecificity of APAO is changed by supplementing either benzaldehyde (R-activating) or pyridoxal (S-activating) with racemic 1-methylspermidine as a substrate [12]. Furthermore, we have characterized earlier the preferred catabolic pathways of symmetrically substituted Spm analogues DESpm and $N^1$,$N^{12}$-dibenzylspermine (DBSpm, *N*,*N'*-bis-(3-benzyl-aminopropyl)butane-1,4-diamine) (Figure 1), and unsymmetrically substituted $N^1$-benzyl-$N^{12}$-ethylspermine (BnEtSpm, *N*-(3-benzyl-aminopropyl)-*N'*-(3-ethylamino-propyl)butane-1,4-diamine) by APAO and SMOX [14]. As protium-deuterium exchange could be used to redirect oxidative metabolism mediated via hydrogen abstraction [1], we synthesized a set of selectively deuterated polyamine analogues (Figure 1) for a proof-of-concept study to alter the catabolic pathways of synthesized analogues and to study the impact of targeted deuteration on the analogues' biological efficacy.

## EXPERIMENTAL PROCEDURES

**Materials.** The synthesis of the analogues is described elsewhere [14, 15]. (*S*)-MeSpd and (*S,S*)-1,12-$Me_2$Spm were prepared as described [16]. The cell lines, DU145, PC3, 22RV1 and MCF7 were obtained from the American Type Culture Collection. Mouse primary embryonic fibroblasts (from C57Bl/6J strain) were isolated as described earlier [17]. [$^{14}$C]-Spm (112 mCi/mmol) was purchased from GE Healthcare. Human recombinant APAO and SMOX were prepared as described earlier [18]. Purified pig kidney DAO and human recombinant MAO-A and MAO-B were from Sigma-Aldrich, and purified bovine plasma SSAO from Worthington. DFMO was supplied by ILEX Oncology Inc.

**Cell culture and sample preparation.** Cells were cultured in high-glucose DMEM supplemented with 10 % heat-inactivated foetal bovine serum, 2 mM *L*-glutamine, 1 mM sodium pyruvate and 50 µg/ml gentamycin (all from Sigma-Aldrich). The cells were plated at a density of $2 \times 10^6$ cells per 10 cm plate, and incubated overnight in a humidified atmosphere at +37 °C, 5 % $CO_2$. The cells were harvested by trypsinization and counted electronically with Coulter Counter model Z1. The cells were washed with PBS, pelleted and stored at −70 °C before analyses. The cells were lysed in a buffer containing 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 % (v/v) Triton X-100, 1 mM dithiothreitol (DTT) and 1 x Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics). Samples were taken for polyamine measurement and the rest of the lysate was centrifuged at 12,000 × g for 20 min at +4 °C and the obtained supernatant was used for enzyme activity assays. Protein concentrations were measured with Bio-Rad protein assay reagent, using dilutions of bovine serum albumin (Bio-Rad) as standards. Cytotoxicity assays were performed on 96-well plates and analysed with Sulforhodamine B staining [19].

**Polyamine and enzyme activity measurements.** Intracellular polyamines and polyamine analogues were measured with HPLC according to a previously published methods [20]. The samples were pre-derivatized with dansyl chloride and processed as described in [21]. An ordinary C-18 column (Hypersil ODS 4.0×250 mm; 5 µm; Agilent Technologies) was used for analytical separation. ODC, AdoMetDC and SSAT activities were measured as previously described [22-24]. SMOX and APAO

activities were measured from cell samples with HPLC by analysing the production of (*S*)-MeSpd from (*S,S*)-1,12-$Me_2$Spm (500 μM) and Put from $N^1$-acetylspermidine (500 μM), respectively. Negative controls included 200 μM MDL 72527.

**Uptake experiments.** Competition experiments with [$^{14}$C]-labelled Spm (10 μM, 25 mCi/mmol) for 10 min were performed as described earlier [24].

**Assays with recombinant/purified enzymes.** Total $H_2O_2$ production was measured with HRP-coupled reaction. The reaction mixture contained 40 units/ml of horse-radish peroxidase (Roche), 1 mM homovanillic acid (Sigma-Aldrich), buffer (100 mM glycine-NaOH pH 9.0 for SMOX and APAO, 50 mM Tris-HCl pH 7.4 for DAO, SSAO and MAO), enzyme (1 μg SMOX and APAO, 1 mg DAO, 100 μg SSAO, 12.5 μg MAO) and 2 mM tested substrate (except for natural substrate 200 μM) in a total volume of 100 μl at 22°C. For inhibition studies, substrates were used at fixed 50 μM concentration. The reaction kinetics were monitored at excitation 315 nm and emission of 420 nm using Envision spectrofluorometer (PerkinElmer). Dilutions of fresh $H_2O_2$ were used as standards. For quantification of analogue metabolites, reactions were performed as described in Table 1 and products analysed with HPLC. Kinetic value determinations with SMOX were carried similarly (using 50, 100, 250 and 500 μM analogue concentrations) as described earlier [14] but the reaction products were determined by using earlier described HPLC-method instead of LC-MS [20].

**Statistical analyses.** The data are expressed as the means ± SD. A software package, GraphPad prism 5.03 (GraphPad software, Inc., San Diego, CA, USA) was used for the analyses of enzyme kinetic data using Michaelis-Menten equation with non-linear fitting (Mr of 55,383 for APAO and Mr of 68,000 for SMOX). One-way ANOVA with Dunnett's post hoc test was used for multiple comparisons (unless otherwise indicated) with aid of GraphPad Prism 5.03 software. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

## RESULTS AND DISCUSSION

***N*-alkylated spermine analogues and their metabolites as substrates of SMOX, APAO, SSAO, DAO and MAO-A/B.** We first screened the deuterated and non-deuterated analogues (Figure 1) and their metabolites as substrates for several oxidases known to metabolize polyamines. Metabolite structures and abbreviations are shown in Supplementary Figure 1. For screening we used coupled horseradish peroxidase (HRP) - homovanillic acid assay system, which measures the total hydrogen peroxide ($H_2O_2$) generation (Figure 2). The results were compared to $H_2O_2$ release from enzymes' natural substrates (Spm, $N^1$-AcSpd, Spm, Put and *p*-tyramine) set as 100 %.

DESpm was the best analogue substrate for human recombinant SMOX, generating 22 % of $H_2O_2$ that was reduced to 4 % with deuterated DESpm-4D (Figure 2). DBSpm and BnEtSpm were both equally good substrates for SMOX. Deuteration at the preferred cleavage sites decreased $H_2O_2$ generation down to 6 % with BnEtSpm-8D and to 7 % with DBSpm-4D(1). Of the analogue metabolites, EtSpm was as good as Spm as a substrate for SMOX, and some reactivity towards BnSpm (15 %) and BnSpd (4 %) were detectable. For human recombinant APAO, deuteration of DBSpm reduced $H_2O_2$ generation to 82 % (DBSpm-8D) and 70 % (DBSpm-4D(1)), respectively (Figure 2). Degradation of BnEtSpm by APAO produced 40 % $H_2O_2$ and unexpectedly only the dideuteration of the benzyl-carbon in the analogue (BnEtSpm-2D(1)) reduced $H_2O_2$ generation to about 18 %. DESpm was the weakest substrate for APAO producing 17 % $H_2O_2$ that diminished to less than 2 % with DESpm-4D. Of the tested analogue metabolites, BnSpd (68 %), BnSpm (64 %) and EtSpm (6 %) were substrates for APAO.

Polyamines and their acetylated derivatives have been shown to be variably metabolized by SSAO, DAO and monoamine oxidases (MAO-A/B). However, to our knowledge, the degradation of *N*-alkylated analogues or their metabolites has not been studied earlier. Only DESpm showed modest substrate properties for purified bovine plasma SSAO, with $H_2O_2$ generation of about 5% in comparison to Spm (Figure 2). Interestingly, mono-*N*-alkylated EtSpm and BnSpm were substrates of SSAO, whereas EtSpd and BnSpd were not degraded although Spd was. Only EtDAP was metabolized by purified pig kidney DAO, generating 30% of $H_2O_2$ in comparison to Put (data not shown). The analogues and their metabolites were very poor substrates for human recombinant MAO-B, all producing <1% of $H_2O_2$ as compared to *p*-tyramine (data not shown). Similarly, they were poor substrates for human recombinant MAO-A, producing <3 % of $H_2O_2$ as compared to *p*-tyramine, with the exception of BnDAP, which showed 23 % $H_2O_2$ generation (data not shown). In summary, the main enzyme responsible for analogue degradation was APAO, followed by SMOX, while SSAO, DAO and MAO-A degraded only some of analogue metabolites having free amino termini.

**Metabolism of *N*-alkylated spermine analogues by human recombinant SMOX and APAO.** To investigate which metabolites were produced by SMOX and APAO and whether deuteration affected the preferred cleavage site, we used HPLC analysis of the reaction mixtures. Degradation of DESpm to EtSpd ($N^4$-exo cleavage) is the main catabolic route by SMOX [14], which was retarded from 20.0 ± 4.2 to 6.6 ± 0.3 μmol/min by deuteration at 3,3- and 10,10-positions (DESpm-4D) when using fixed 0.5 mM substrate concentration (Table 1). In addition, deuteration increased DESpm to EtDAP production ($N^4$-endo cleavage).

Degradation of BnEtSpm to EtSpd is the main metabolic route by SMOX [14], which was dramatically slowed down with BnEtSpm-4D and BnEtSpm-8D, respectively, but increased with BnEtSpm-2D(1) (Table 1). In addition, generation of BnSpd from BnEtSpm-8D was reduced. DBSpm was degraded as efficiently to BnSpm as to BnSpd ($N^1$/$N^4$-exo cleavages, respectively) by SMOX (Table 1). Symmetrical exo-$N^{1,1}$-dideuteration (DBSpm-4D(1)) retarded $V_{max}$ of debenzylation of DBSpm to BnSpm ($K_m$ 117 μM, $V_{max}$ 19.0 vs $K_m$ 88.1 μM, $V_{max}$ 4.2). Interestingly, extensive dideuteration at 2,2- and 3,3-positions (DBSpm-8D) increased analogue affinity ($K_m$ 40.9 μM) and slightly increased velocity ($V_{max}$ 21.5) of DBSpm to BnSpm conversion. Similarly, DBSpm-8D degradation to BnSpd was retarded by 4-fold in comparison to DBSpm with minor change in affinity ($K_m$ 24.7 μM, $V_{max}$ 13.4 vs $K_m$ 30.1 μM, $V_{max}$ 3.5).

In contrast to SMOX, APAO strongly prefers $N^4$-exo cleavage of DBSpm over $N^1$-exo cleavage [14]. With DBSpm-8D, BnSpd generation was reduced while BnSpm production was increased (Table 1). By contrast, symmetrical exo-$N^{1,1}$-dideuteration (DBSpm-4D(1)) increased BnSpd production and decreased BnSpm production. DESpm to EtSpd conversion by APAO was markedly reduced with DESpm-4D, while DESpm to EtSpm conversion was increased. BnEtSpm to EtSpd conversion was reduced with both BnEtSpm-4D and -8D, while generation of EtSpm wash highly increased. By contrast, BnEtSpm-2D(1) to EtSpm generation was slowed down. With both enzymes, BnEtSpm-2D(2) and DBSpm-4D(2) were degraded like their non-deuterated counterparts.

Taken together, these results show that by targeted deuteration it is possible to slow down analogue catabolism and control the preferred cleavage site by APAO and SMOX.

***N*-alkylated spermine analogues and their metabolites as inhibitors of SSAO, DAO, APAO and SMOX.** We next studied those analogues and metabolites with poor substrate properties as inhibitors of the enzymes, using 50 μM of substrate and 50 and 500 μM inhibitor concentrations (Figure 3). SSAO was inhibited especially by BnSpd and by DBSpm and EtSpd, whereas DAO showed dramatic inhibition to EtSpd and BnSpd. EtSpd was the most efficient inhibitor of APAO, while SMOX was more resistant towards the tested compounds, showing ~50% inhibition with 500 μM EtSpd, BnSpd and Spd.

APAO is constitutively expressed and poorly inducible. In contrast, SMOX is inducible enzyme by various stimuli like inflammation and the presence of polyamine analogues [25, 26]. The oxidation of Spm by SMOX produces a by-product 3-aminopropanal which spontaneously decomposes to highly toxic acrolein, whereas degradation via SSAT/APAO produces non-cytotoxic 3-acetoamidopropanal [27]. Increased SMOX activity has been associated with prostate, colon and *Helicobacteria pylori*-induced gastric carcinogenesis [28]. SSAO is involved in neovascularization and its inhibition reduces tumour growth in mice [29]. The role of DAO in cancer is not as clear, as both increased and decreased activities have been reported depending on cancer type [30, and references therein]. It has been proposed that DAO restrains cancer growth by degrading Put. On the other hand, the action of DAO produces energy from Put via tricarboxylic acid cycle through a so-called GABA shunt. Copper chelating agents such as tetrathiomolybdate or polyamine analogue triethylenetetramine inhibit both SSAO and DAO and exhibit anticancer potency [31]. Thus, by selective deuteration of polyamine analogue, it may be possible to widen drug target profile *in vivo*, e.g. enhance production of bioactive metabolites (such as BnSpd) that inhibit SSAO/DAO activity.

**Uptake competition of analogues with Spm in DU145 cells.** The polyamine transport system has been long recognized as a feasible target for drug design, either for transporting different cargoes inside the cells or for developing inhibitors which prevent polyamine uptake from the circulation [32]. The genes associated with polyamine transport in mammalian cells have not yet been cloned [33]. However, substantial experimental data indicates that polyamines are actively taken up via saturable, ATP-dependent system [34, 35]. In addition, some di(benzyl)polyamine analogues having extended carbon backbone and possessing antimalarial potency are substrates for a mammalian cell-transport system which is distinct from the polyamine transport system [36]. These analogues are substrates of rat liver APAO [37].

Since extensive deuteration, especially with lipophilic *N*-terminal substituent, may decrease the affinity for the transport, we investigated whether deuteration affected the uptake of the *N*-alkylated analogues by polyamine transporter in DU145 prostate carcinoma cells. As indicated in Figure 4, all the analogues competed with [$^{14}$C]-labelled Spm for polyamine transport in 10-min competition experiments. The best competitor with Spm was unsymmetrically substituted BnEtSpm, showing no affinity difference with deuterated and non-deuterated isoforms. Likewise, deuteration of DESpm did not markedly affect its ability to compete for uptake with Spm. However, heavily deuterated DBSpm-8D had lower affinity for the transport than its non-deuterated counterpart. Thus, it may be possible to alter the affinity for the polyamine transport system through deuteration of the carbon chain of polyamine analogue.

***N*-alkylated spermine analogues as polyamine antimetabolites and the effect of deuteration on their biological efficacy.** We next investigated how deuteration affected the biological efficacy of the analogues in DU145 cells. As depicted in Figure 5, DESpm suppressed the polyamine biosynthetic

enzymes ODC and *S*-adenosyl-*L*-methionine decarboxylase (AdoMetDC; official name AMD1), induced spermidine/spermine-$N^1$-acetyltransferase (SSAT; official name SAT1) and depleted intracellular polyamine pools (Table 2) in DU145 cells in 1-3 days more efficiently than the other analogues. Interestingly, the most efficient antiproliferative effect in DU145 cells was accomplished with BnEtSpm, although Spd and Spm pools were not nearly as efficiently depleted as with DESpm. However, BnEtSpm was the most efficient analogue in inducing SMOX activity, which correlated highly with its antiproliferative activity (Figure 5). Surprisingly, no clear differences in the growth inhibition were observed between deuterated and non-deuterated analogues of DESpm and BnEtSpm, while DBSpm-8D was slightly less growth-inhibitory than its non-deuterated isoform. It should be noted that longer treatment with DESpm, which completely depleted Spd pools in 3 days, would most likely lead to complete cytostasis since Spd is the only natural precursor of hypusine, which is an essential part of active eukaryotic translation initiation factor 5A (eIF5A) that is needed to sustain proliferation [38].

We also investigated the biological effects of the analogues in wild-type mouse primary embryonic fibroblasts (MEFs) used as non-cancerous reference cells. These cells were more resistant to the analogue-mediated growth inhibition, and therefore higher concentrations (50 μM) of the analogues were used. Interestingly, DBSpm was the most cytotoxic analogue in MEFs (Figure 6C). When BnEtSpm and DBSpm analogues were used at concentrations close to their $IC_{50}$ values (100 and 10 μM, respectively) for 2 days, DBSpm-8D showed less cytotoxicity than its parent non-deuterated analogue, while BnEtSpm-2D(1) was more cytotoxic (Figure 6E). Analysis of basal APAO and SMOX activities revealed that MEFs had higher SMOX and APAO activities than DU145 cells (Figure 6F), which likely explains the observed kinetic isotope effect on the antiproliferative efficacy of the analogues in MEFs. Like in DU145 cells, the antiproliferative activity correlated with the induction of SMOX activity (Figure 6B, C), but not with SSAT activity (Figure 6A). We also found similar correlations with growth inhibition and SMOX activity, but not with SSAT activity or total Spd + Spm level, also in PC3 and 22RV1 prostate carcinoma and in MCF7 breast carcinoma cell lines, in which BnEtSpm was the most cytotoxic analogue (Supplementary Figure 2).

We then investigated the cytotoxicity of analogue metabolites in DU145 cells, and whether growth inhibition by analogues or their metabolites could be prevented by co-treatment with SC or MDL 72527 (Figure 7). SC inhibits amine oxidases present in the foetal bovine serum that act on free primary amine groups, producing $H_2O_2$ and reactive aldehyde species as by-products. SC also inhibits cell membrane-bound form of SSAO, also known as VAP-1 [39]. As shown in Figure 7, none of the tested metabolites was toxic at 10 μM concentration. At 100 μM, EtSpm and BnSpm had dramatic antiproliferative effect in DU145 cells, while BnSpd and BnDAP had some but less prominent antiproliferative effect. Cotreatment with 1 mM SC completely prevented growth inhibition caused by EtSpm, but only partially that caused by BnSpm. By contrast, SC potentiated the growth inhibition of BnSpd and BnEtSpm.

At the high concentration (250 μM), even as a single agent, MDL 72527 had antiproliferative effect in DU145 cells (Figure 7), possibly due to its known lysosomotropic properties [40]. MDL 72527 partially reversed the growth-inhibitory effect of BnEtSpm, whereas it dramatically potentiated the cytotoxicity of DBSpm. At low concentration (10 μM), it had less effect on BnEtSpm or DBSpm efficacy, but it partially reversed growth inhibition caused by DESpm. It also prevented the moderate growth inhibitory effects of BnSpd and BnDAP. It should be noted that the interpretation of these results is complicated by the fact that MDL 72527 may compete with the analogues for uptake. In

addition, DBSpm might be taken up also via other transporter(s) than polyamine transporter, as demonstrated for some bis(benzyl)polyamine analogues having extended carbon backbone [36]. Furthermore, by using coupled HRP-assay system we found that MDL 72527 (100 μM) inhibits also SSAO and DAO *in vitro* (data not shown), but this has not yet been investigated in cell culture.

Earlier studies have aimed at solving the mechanisms of action of *N*-alkylated polyamine analogues in inducing apoptosis, cell toxicity and cessation of cell growth [41, 42]. DESpm has been shown to suppress ODC and AdoMetDC and induce SSAT and SMOX, thus efficiently depleting endogenous polyamines [41]. Several studies have shown that induction of SSAT leads to cessation of cell growth or induction of apoptosis [43]. SSAT-deficient mouse embryonic stem cells were more resistant [44] while SSAT overexpressing MEFs were more sensitive to DENSpm than their wild-type counterparts [17]. Our present study shows that kinetic isotope effect in *N*-alkylated polyamine analogues was clearly detectable with SMOX and APAO *in vitro*. We detected 3-6-fold reduction in the reaction velocity and moderate effect on affinity ($K_m$) with the deuterated analogues in comparison to their non-deuterated counterparts. However, their biological efficacy in DU145 cells remained almost the same regardless of deuteration, while in MEFs, which had higher basal SMOX and APAO activities, deuteration affected more on analogue cytotoxicity. Thus, analogue catabolism does not seem to be the major determinant of its cytotoxicity in those cell lines where APAO and SMOX activities are low. Importantly, we found that the ability of the analogues to induce SMOX varied between cell lines, but it seemed to be a consistent determinant of the analogues' antiproliferative action.

In conclusion, targeted deuteration not only slows down *N*-alkylpolyamines' metabolism but also allows "metabolic switching", offering means for controlling their primary metabolism, e.g. diminish debenzylation, and enhance DBSpm to BnSpd, and BnEtSpm to EtSpd degradation. BnSpd and EtSpd, in contrast to BnSpm and EtSpm, were found to be potent inhibitors of DAO and SSAO *in vitro*. Our present data opens interesting avenues for *in vivo* characterization of novel *N*-alkylated polyamine analogues.

## SUPPORTING INFORMATION

Supplementary Figures S1-S2.


## ACKNOWLEDGEMENTS

The authors thank Ms. Tuula Reponen, Ms. Anne Karppinen and Ms. Maritta Salminkoski for skilful technical assistance.


## DECLARATIONS OF INTEREST

The authors declare no conflicts of interest.


## FUNDING INFORMATION

The research was supported by grants from the Academy of Finland and by the strategic funding from the University of Eastern Finland.


## AUTHOR CONTRIBUTION STATEMENT

M.R.H., A.L.-A. and J.V. synthesised chemicals and analysed their purity. S.U., A.-L.A., M.T.H. and T.A.K. performed the experiments and analysed data. M.T.H., L.A. and T.A.K. planned the experiments. S.U. and M.T.H. prepared the figures. All authors participated in writing of the manuscript.

## REFERENCES


1 Gant, T. G. (2014) Using deuterium in drug discovery: leaving the label in the drug. J. Med. Chem. **57**, 3595-3611

2 Krumbiegel, P. (2011) Large deuterium isotope effects and their use: a historical review. Isot. Env. Health Stud. **47**, 1-17

3 Timmins, G. S. (2014) Deuterated drugs: where are we now? Exp. Opin. Therap. Pat. **24**, 1067-1075

4 Pegg, A. E. (2009) Mammalian polyamine metabolism and function. IUBMB life. **61**, 880-894

5 Seiler, N. (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. Curr. Drug Targ. **4**, 537-564

6 Casero, R. A., Jr. and Marton, L. J. (2007) Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. Drug discov. **6**, 373-390

7 Seiler, N. (2003) Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 2. Structural analogues and derivatives. Curr. Drug Targets. **4**, 565-585

8 Bergeron, R. J., Feng, Y., Weimar, W. R., McManis, J. S., Dimova, H., Porter, C., Raisler, B. and Phanstiel, O. (1997) A comparison of structure-activity relationships between spermidine and spermine analogue antineoplastics. J. Med. Chem. **40**, 1475-1494

9 Bergeron, R. J., Weimar, W. R., Luchetta, G., Streiff, R. R., Wiegand, S. J., Perrin, J., Schreier, K. M., Porter, C., Yao, G. W. and Dimova, H. (1995) Metabolism and pharmacokinetics of N1, N11-diethylnorspermine. Drug Metab. Dispos. **23**, 1117-1125

10 Lawson, K. R., Marek, S., Linehan, J. A., Woster, P. M., Casero, R. A., Jr., Payne, C. M. and Gerner, E. W. (2002) Detoxification of the polyamine analogue N1-ethyl-N11-[(cycloheptyl)methy]-4,8-diazaundecane (CHENSpm) by polyamine oxidase. Clin. Cancer Res. **8**, 1241-1247

11 Bitonti, A. J., Bush, T. L. and McCann, P. P. (1989) Regulation of polyamine biosynthesis in rat hepatoma (HTC) cells by bisbenzyl polyamine analogue. Biochem. J. **257**, 769-774

12 Järvinen, A., Keinänen, T. A., Grigorenko, N. A., Khomutov, A. R., Uimari, A., Vepsäläinen, J., Närvänen, A., Alhonen, L. and Jänne, J. (2006) Guide molecule-driven stereospecific degradation of alpha-methylpolyamines by polyamine oxidase. J. Biol. Chem. **281**, 4589-4595

13 Hyvönen, M. T., Keinänen, T. A., Cerrada-Gimenez, M., Sinervirta, R., Grigorenko, N., Khomutov, A. R., Vepsäläinen, J., Alhonen, L. and Jänne, J. (2007) Role of hypusinated eukaryotic translation initiation factor 5A in polyamine depletion-induced cytostasis. J. Biol. Chem. **282**, 34700-34706

14 Häkkinen, M. R., Hyvönen, M. T., Auriola, S., Casero, R. A., Jr., Vepsäläinen, J., Khomutov, A. R., Alhonen, L. and Keinänen, T. A. (2010) Metabolism of N-alkylated spermine analogues by polyamine and spermine oxidases. Amino acids. **38**, 369-381

15 Häkkinen, M. R., Keinänen, T. A., Khomutov, A. R., Auriola, S., Weisell, J., Alhonen, L., Jänne, J. and Vepsäläinen, J. (2009) Synthesis of novel deuterium labelled derivatives of N-alkylated polyamines. Tetrahedron. **65**, 547-562

16 Grigorenko, N. A., Khomutov, A. R., Keinänen, T. A., Järvinen, A., Alhonen, L., Jänne, J. and Vepsäläinen, J. (2007) Synthesis of novel optical isomers of alpha-methylpolyamines. Tetrahedron. **63**, 2257-2262

17 Alhonen, L., Karppinen, A., Uusi-Oukari, M., Vujcic, S., Korhonen, V.-P., Halmekytö, M., Kramer, D. L., Hines, R., Jänne, J. and Porter, C. W. (1998) Correlation of polyamine and growth responses to $N^{1}$,$N^{11}$-diethylnorspermine in primary fetal fibroblasts derived from transgenic mice overexpressing spermidine/spermine $N^{1}$-acetyltransferase. J. Biol. Chem. **273**, 1964-1969

18 Järvinen, A., Grigorenko, N., Khomutov, A. R., Hyvönen, M. T., Uimari, A., Vepsäläinen, J., Sinervirta, R., Keinänen, T. A., Vujcic, S., Alhonen, L., Porter, C. W. and Jänne, J. (2005) Metabolic stability of alpha -methylated polyamine derivatives and their use as substitutes for the natural polyamines. J. Biol. Chem. **280**, 6595-6601
19 Orellana, E. A. and Kasinski, A. L. (2016) Sulforhodamine B (SRB) Assay in Cell Culture to Investigate Cell Proliferation. Bio-protocol. **6**
20 Hyvönen, T., Keinänen, T. A., Khomutov, A. R., Khomutov, R. M. and Eloranta, T. O. (1992) Monitoring of the uptake and metabolism of aminooxy analogues of polyamines in cultured cells by high-performance liquid chromatography. J. Chromatogr. **574**, 17-21
21 Kabra, P. M. and Lee, H. K. (1986) Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J. Chromatogr. **380**, 19-32
22 Jänne, J. and Williams-Ashman, H. G. (1971) On the purification of L-ornithine decarboxylase from rat prostate and effects of thiol compounds on the enzyme. J. Biol. Chem. **246**, 1725-1732
23 Libby, P. R., Ganis, B., Bergeron, R. J. and Porter, C. W. (1991) Characterization of human spermidine/spermine N1-acetyltransferase purified from cultured melanoma cells. Arch. Biochem. Biophys. **284**, 238-244
24 Hyvönen, M. T., Howard, M. T., Anderson, C. B., Grigorenko, N., Khomutov, A. R., Vepsäläinen, J., Alhonen, L., Jänne, J. and Keinänen, T. A. (2009) Divergent regulation of the key enzymes of polyamine metabolism by chiral alpha-methylated polyamine analogues. Biochem. J. **422**, 321-328
25 Wang, Y., Devereux, W., Woster, P. M., Stewart, T. M., Hacker, A. and Casero, R. A., Jr. (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. Cancer Res. **61**, 5370-5373.
26 Babbar, N., Murray-Stewart, T. and Casero, R. A., Jr. (2007) Inflammation and polyamine catabolism: the good, the bad and the ugly. Biochem. Soc. Trans. **35**, 300-304
27 Wood, P. L., Khan, M. A. and Moskal, J. R. (2007) The concept of "aldehyde load" in neurodegenerative mechanisms: cytotoxicity of the polyamine degradation products hydrogen peroxide, acrolein, 3-aminopropanal, 3-acetamidopropanal and 4-aminobutanal in a retinal ganglion cell line. Brain Res. **1145**, 150-156
28 Battaglia, V., DeStefano Shields, C., Murray-Stewart, T. and Casero, R. A., Jr. (2014) Polyamine catabolism in carcinogenesis: potential targets for chemotherapy and chemoprevention. Amino acids. **46**, 511-519
29 Li, R., Li, H., Luo, H. J., Lin, Z. X., Jiang, Z. W. and Luo, W. H. (2013) SSAO inhibitors suppress hepatocellular tumor growth in mice. Cellular immunology. **283**, 61-69
30 Seiler, N. (2004) Catabolism of polyamines. Amino acids. **26**, 217-233
31 Hyvönen, M. T., Ucal, S., Pasanen, M., Peräniemi, S., Weisell, J., Khomutov, M., Khomutov, A. R., Vepsäläinen, J., Alhonen, L. and Keinänen, T. A. (2016) Triethylenetetramine modulates polyamine and energy metabolism and inhibits cancer cell proliferation. Biochem. J. **473**, 1433-1441
32 Muth, A., Madan, M., Archer, J. J., Ocampo, N., Rodriguez, L. and Phanstiel, O. t. (2014) Polyamine transport inhibitors: design, synthesis, and combination therapies with difluoromethylornithine. J. Med. Chem. **57**, 348-363
33 Abdulhussein, A. A. and Wallace, H. M. (2014) Polyamines and membrane transporters. Amino acids. **46**, 655-660
34 Igarashi, K. and Kashiwagi, K. (2010) Characteristics of cellular polyamine transport in prokaryotes and eukaryotes. Plant. Physiol. Biochem. **48**, 506-512
35 Poulin, R., Casero, R. A. and Soulet, D. (2012) Recent advances in the molecular biology of metazoan polyamine transport. Amino acids. **42**, 711-723

36 Byers, T. L., Bitonti, A. J. and McCann, P. P. (1990) Bis(benzyl)polyamine analogues are substrates for a mammalian cell-transport system which is distinct from the polyamine-transport system. Biochem. J. **269**, 35-40
37 Bitonti, A. J., Dumont, J. A., Bush, T. L., Stemerick, D. M., Edwards, M. L. and McCann, P. P. (1990) Bis(benzyl)polyamine analogs as novel substrates for polyamine oxidase. J. Biol. Chem. **265**, 382-388
38 Park, M. H., Wolff, E. C. and Folk, J. E. (1993) Hypusine: its post-translational formation in eukaryotic initiation factor 5A and its potential role in cellular regulation. BioFactors. **4**, 95-104
39 Bono, P., Salmi, M., Smith, D. J. and Jalkanen, S. (1998) Cloning and characterization of mouse vascular adhesion protein-1 reveals a novel molecule with enzymatic activity. J. Immunol. **160**, 5563-5571
40 Dai, H. Q., Kramer, D. L., Yang, C. Y., Murti, K. G., Porter, C. W. and Cleveland, J. L. (1999) The polyamine oxidase inhibitor MDL-72,527 selectively induces apoptosis of transformed hematopoietic cells through lysosomotropic effects. Cancer Res. **59**, 4944-4954
41 Huang, Y., Pledgie, A., Casero, R. A., Jr. and Davidson, N. E. (2005) Molecular mechanisms of polyamine analogs in cancer cells. Anti-cancer drugs. **16**, 229-241
42 Albanes, L., Bergeron, R. J. and Pegg, A. E. (1993) Investigations on the mechanisms by which mammalian cell growth is inhibited by N1N12-bis(ethyl)spermine. Biochem. J. **291**, 131-137
43 Pegg, A. E. (2008) Spermidine/spermine-N(1)-acetyltransferase: a key metabolic regulator. Am. J. Physiol Endocrinol Metab. **294**, E995-1010
44 Niiranen, K., Pietilä, M., Pirttilä, T. J., Järvinen, A., Halmekytö, M., Korhonen, V. P., Keinänen, T. A., Alhonen, L. and Jänne, J. (2002) Targeted disruption of spermidine/spermine N1-acetyltransferase gene in mouse embryonic stem cells. Effects on polyamine homeostasis and sensitivity to polyamine analogues. J. Biol. Chem. **277**, 25323-25328

FIGURE LEGENDS

Figure 1. Structures of the natural polyamine Spm and its analogues used in the study.

Figure 2. The analogues and their metabolites as substrates for (A) SMOX, (B) APAO, and (C) SSAO. The compounds (2 mM) were tested in coupled HRP-homovanillic acid assay system, which measures total $H_2O_2$ production. Natural substrates were used at 200 μM. The activities for natural substrates were SMOX (spermine) 6318 ± 341 nmol/min/mg, APAO ($N^1$-AcSpd) 5547 ± 136 nmol/min/mg, and SSAO (spermine) 4.5 ± 0.2 nmol/min/mg. Data are means ± S.D., n = 3. Statistical significance ***, $p < 0.001$ as compared to non-deuterated parent analogue.

Figure 3. Inhibition of (A) SSAO, (B) DAO, (C) APAO, and (D) SMOX with analogs and their metabolites. Substrate (Spm, Put, $N^1$-AcSpd and Spm, respectively) concentration was 50 μM for each enzyme, and inhibitors were tested at 50 and 500 μM. The activities for natural substrates were SSAO (spermine) 3.7 ± 0.1 nmol/min/mg, DAO (putrescine) 109 ± 5 nmol/min/mg, APAO ($N^1$-AcSpd) 1770 ± 91 nmol/min/mg, and SMOX (spermine) 2350 ± 130 nmol/min/mg. Data are means ± S.D, n = 3. Statistical significance **, $p<0.01$; ***, $p<0.001$ as compared to control sample (100%, no inhibitor).

Figure 4. Analogue competition for uptake with 10 μM [$^{14}$C]-labeled Spm in DU145 cells. Data are means ± S.D., n = 3. Statistical significance *, $p<0.05$; ***, $p<0.001$ as compared to non-deuterated parent analogue.

Figure 5. Effect of the analogues on (A) ODC activity, (B) AdoMetDC activity, (C) SSAT activity, (D) SMOX activity, and (E) growth of DU145 prostate cancer cells. The cells were treated with 25 μM analogues for 1, 2 or 3 days. Figure (F) shows the correlation between growth and SMOX activity. Data are means ± S.D., n = 3. Statistical significance **, $p<0.01$ as compared to non-deuterated parent analogue.

Figure 6. Effect of the analogues on (A) SSAT activity, (B) SMOX activity, and (C) growth of MEFs. The cells were treated with 50 μM analogues for 1, 2 or 3 days. Figure (D) shows the correlation between growth and SMOX activity. Data are means ± S.D. of triplicate culture plates. (E) Effect of variably deuterated 100 μM BnEtSpm and 10 μM DBSpm on MEF growth (2d) determined by Sulforhodamine B staining (n = 6). (F) Basal SMOX and APAO activities in DU145 and MEF cells (n = 3). Statistical significance *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ as compared to non-deuterated parent analogue.

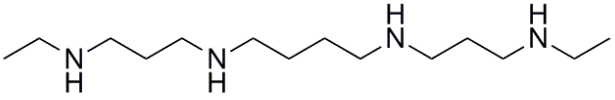
Figure 7. Effect of (A) analogues (25 μM), and (B) their metabolites (10 or 100 μM) on the growth of DU145 cells. The cells were treated for 2 days without or with SC (1 mM) or MDL 72527 (10 or 250 μM). Data are means ± S.D., n = 6. Statistical significance markings are omitted for clarity.

Table 1: Degradation of analogues by human recombinant SMOX and APAO. Analogues (100 μM) were incubated with 0.5 μg of enzyme for 30 min (SMOX) or 15 min (APAO) at +37°C, reaction stopped with the addition of sulfosalicylic acid with diaminoheptane, and reaction products analysed with HPLC. n.d., not detectable. Data are means ± S.D., n = 3. Reference activity for SMOX (Spm) was 396 ± 4 μmol/min/μmol and for APAO ($N^1$-AcSpd) was 217 ± 14 μmol/min/μmol.

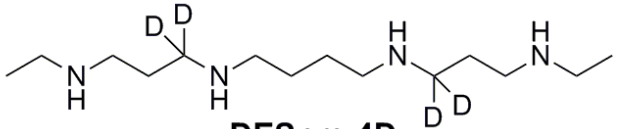
| | Put | Spd | EtDAP | EtSpd | EtSpm | BnDAP | BnSpd | BnSpm | $BnNH_2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *(μmol/min/μmol)* | | | | | |
| | | | | *SMOX* | | | | | |
| DESpm | | 1.0 ± 0.0 | 0.7 ± 0.1 | 20.0 ± 4.4 | | | | | |
| DESpm-4D | | 0.5 ± 0.1 | 1.2 ± 0.0 | 6.6 ± 0.3 | | | | | |
| BnEtSpm | | 5.8 ± 0.1 | 0.5 ± 0.0 | 6.8 ± 0.1 | 0.7 ± 0.0 | n.d. | 3.0 ± 0.1 | | 4.2 ± 0.1 |
| BnEtSpm-4D | | 6.9 ± 0.1 | 0.6 ± 0.0 | 0.9 ± 0.0 | 0.7 ± 0.0 | n.d. | 3.9 ± 0.1 | | n.d. |
| BnEtSpm-8D | | 6.7 ± 0.1 | 0.5 ± 0.0 | 1.0 ± 0.0 | 1.5 ± 0.0 | n.d. | 0.6 ± 0.0 | | n.d. |
| BnEtSpm-2D(1) | | 1.5 ± 0.1 | 0.6 ± 0.0 | 7.4 ± 0.1 | 0.5 ± 0.0 | n.d. | 3.7 ± 0.0 | | 4.9 ± 0.1 |
| BnEtSpm-2D(2) | | 6.0 ± 0.0 | 0.5 ± 0.0 | 6.8 ± 0.2 | 0.7 ± 0.0 | n.d. | 3.2 ± 0.0 | | 4.4 ± 0.0 |
| DBSpm | | 8.3 ± 0.3 | | | | 1.1 ± 0.0 | 5.7 ± 0.2 | 5.7 ± 0.1 | 8.6 ± 0.3 |
| DBSpm-8D | | 4.3 ± 0.2 | | | | 1.4 ± 0.0 | 1.3 ± 0.1 | 13.0 ± 0.1 | 1.2 ±0.1 |
| DBSpm-4D(1) | | 2.9 ± 0.0 | | | | 2.0 ± 0.0 | 9.6 ± 0.2 | 0.7 ± 0.0 | 7.2 ± 0.1 |
| DBSpm-4D(2) | | 7.5 ± 0.4 | | | | 1.3 ± 0.0 | 5.8 ± 0.1 | 5.3 ± 0.1 | 7.8 ± 0.2 |
| | | | | *APAO* | | | | | |
| DESpm | | 3.4 ± 0.5 | 4.9 ± 0.6 | 42.1 ± 5.2 | 0.5 ± 0.2 | | | | |
| DESpm-4D | | 1.2 ± 0.2 | 7.4 ± 0.4 | 12.9 ± 1.0 | 3.8 ± 0.1 | | | | |
| BnEtSpm | | n.d. | 3.1 ± 0.1 | 46.1 ± 0.8 | 3.0 ± 0.1 | | | | 5.6 ± 0.2 |
| BnEtSpm-4D | | n.d. | 2.7 ± 0.0 | 32.4 ± 0.6 | 15.9 ± 0.2 | | | | n.d. |
| BnEtSpm-8D | | n.d. | 2.5 ± 0.0 | 32.4 ± 0.7 | 15.4 ± 0.4 | | | | n.d. |
| BnEtSpm-2D(1) | | 2.3 ± 0.1 | 1.9 ± 0.1 | 38.5 ± 0.4 | 0.7 ± 0.3 | | | | 10.8 ± 0.1 |
| BnEtSpm-2D(2) | | 2.2 ± 0.1 | 3.2 ± 0.1 | 45.7 ± 1.2 | 3.1 ± 0.1 | | | | 5.1 ± 0.1 |
| DBSpm | 1.5 ± 0.1 | 23.1 ± 0.5 | | | | 1.2 ± 0.0 | 48.7 ± 1.0 | 3.7 ± 0.1 | 14.1 ± 0.3 |
| DBSpm-8D | n.d. | 14.8 ± 0.3 | | | | 1.2 ± 0.1 | 30.0 ± 0.3 | 11.8 ± 0.2 | n.d. |
| DBSpm-4D(1) | n.d. | 8.2 ± 0.2 | | | | 0.8 ± 0.0 | 70.1 ± 1.3 | 0.5 ± 0.2 | 26.2 ± 0.6 |
| DBSpm-4D(2) | n.d. | 23.2 ± 0.9 | | | | 1.2 ± 0.1 | 50.6 ± 0.7 | 2.5 ± 0.1 | 12.8 ± 0.2 |

Table 2. Intracellular polyamine pools in DU145 cells cultured with 25 μM analogues for 1-3 days. n.d, not detectable; NA, not analysed. Data are means ± S.D., n = 3.

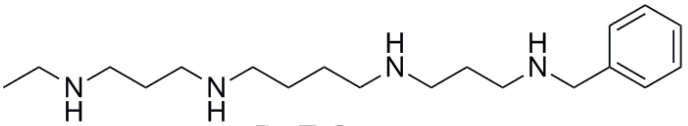
| | Put | Spd | Spm | $N^1$-AcSpd | Analogue |
|---|---|---|---|---|---|
| | | *(pmol/$10^6$ cells)* | | | |
| | | *1d* | | | |
| Control | 457±81 | 2024±109 | 1754±102 | n.d. | |
| DESpm | n.d. | 129±7 | 212±56 | 61±25 | NA |
| DESpm-4D | n.d. | 92±22 | 194±14 | 83±8 | NA |
| BnEtSpm | 71±62 | 396±42 | 1269±113 | 66±26 | NA |
| BnEtSpm-4D | 79±77 | 480±49 | 1263±213 | 55±32 | NA |
| BnEtSpm-8D | 66±8 | 419±24 | 1053±214 | 70±35 | NA |
| DBSpm | 165±165 | 1401±199 | 2060±447 | <10 | NA |
| DBSpm-8D | 180±48 | 1387±75 | 2299±124 | n.d. | NA |
| | | *2d* | | | |
| Control | 217±11 | 1593±69 | 1502±151 | <10 | |
| DESpm | 57±12 | 62±40 | 60±19 | n.d. | NA |
| DESpm-4D | n.d. | 32±2 | 39±3 | n.d. | NA |
| BnEtSpm | 85±34 | 347±23 | 584±34 | 37±12 | NA |
| BnEtSpm-4D | n.d. | 392±35 | 545±121 | 117±21 | NA |
| BnEtSpm-8D | n.d. | 381±30 | 595±44 | 89±26 | NA |
| DBSpm | 165±28 | 1187±45 | 1729±93 | 18±11 | NA |
| DBSpm-8D | 136±18 | 1150±104 | 1627±165 | <10 | NA |
| | | *3d* | | | |
| Control | 88±19 | 1221±29 | 1115±65 | <10 | |
| DESpm | 48±16 | n.d. | 31±2 | n.d. | 930±97 |
| DESpm-4D | n.d. | n.d. | 36±11 | n.d. | 821±25 |
| BnEtSpm | n.d. | 273±83 | 473±95 | 63±31 | 103±18 |
| BnEtSpm-4D | n.d. | 291±44 | 389±113 | 46±16 | NA |
| BnEtSpm-8D | n.d. | 276±51 | 330±90 | 63±41 | 87±8 |
| DBSpm | 111±22 | 1000±27 | 1476±82 | 18±4 | 259±14 |
| DBSpm-8D | 77±5 | 916±6 | 1447±12 | 31±2 | 362±40 |



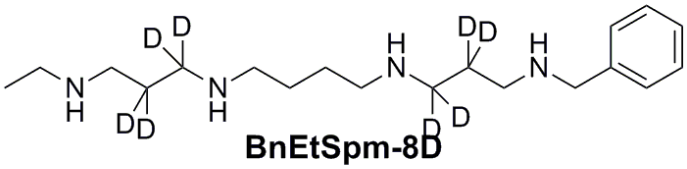
**DESpm**



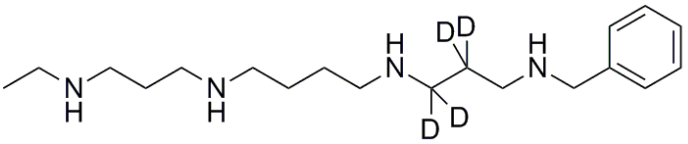
**DESpm-4D**



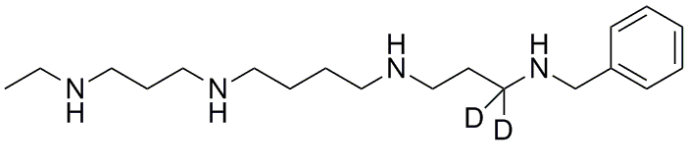
**BnEtSpm**



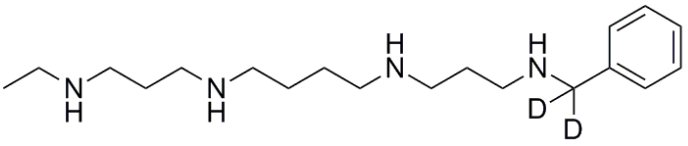
**BnEtSpm-8D**



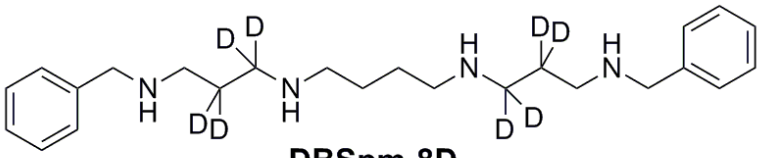
**BnEtSpm-4D**



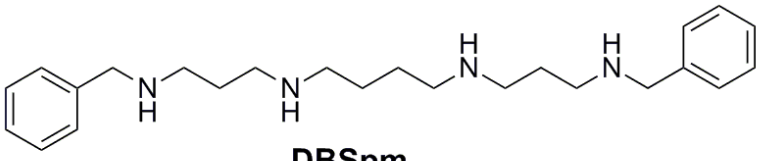
**BnEtSpm-2D (2)**



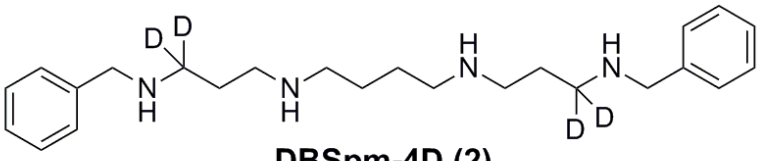
**BnEtSpm-2D (1)**



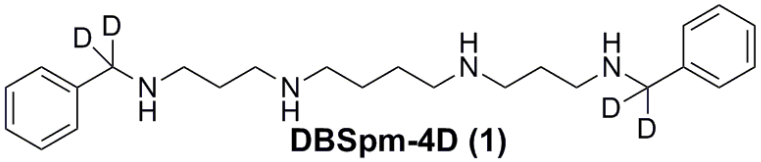
**DBSpm-8D**



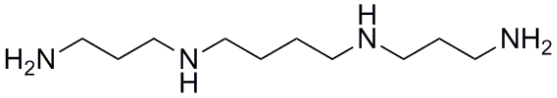
**DBSpm**



**DBSpm-4D (2)**

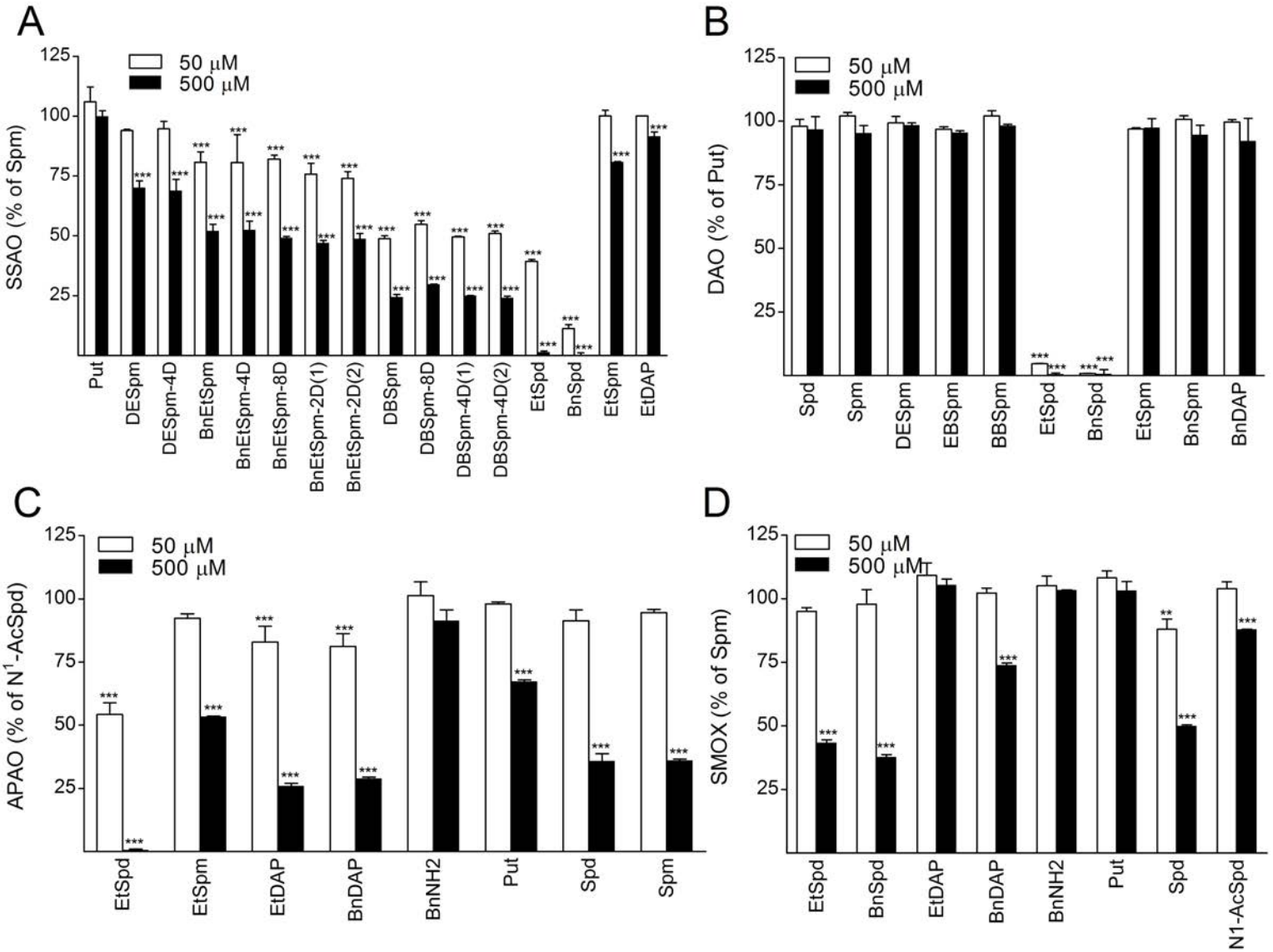


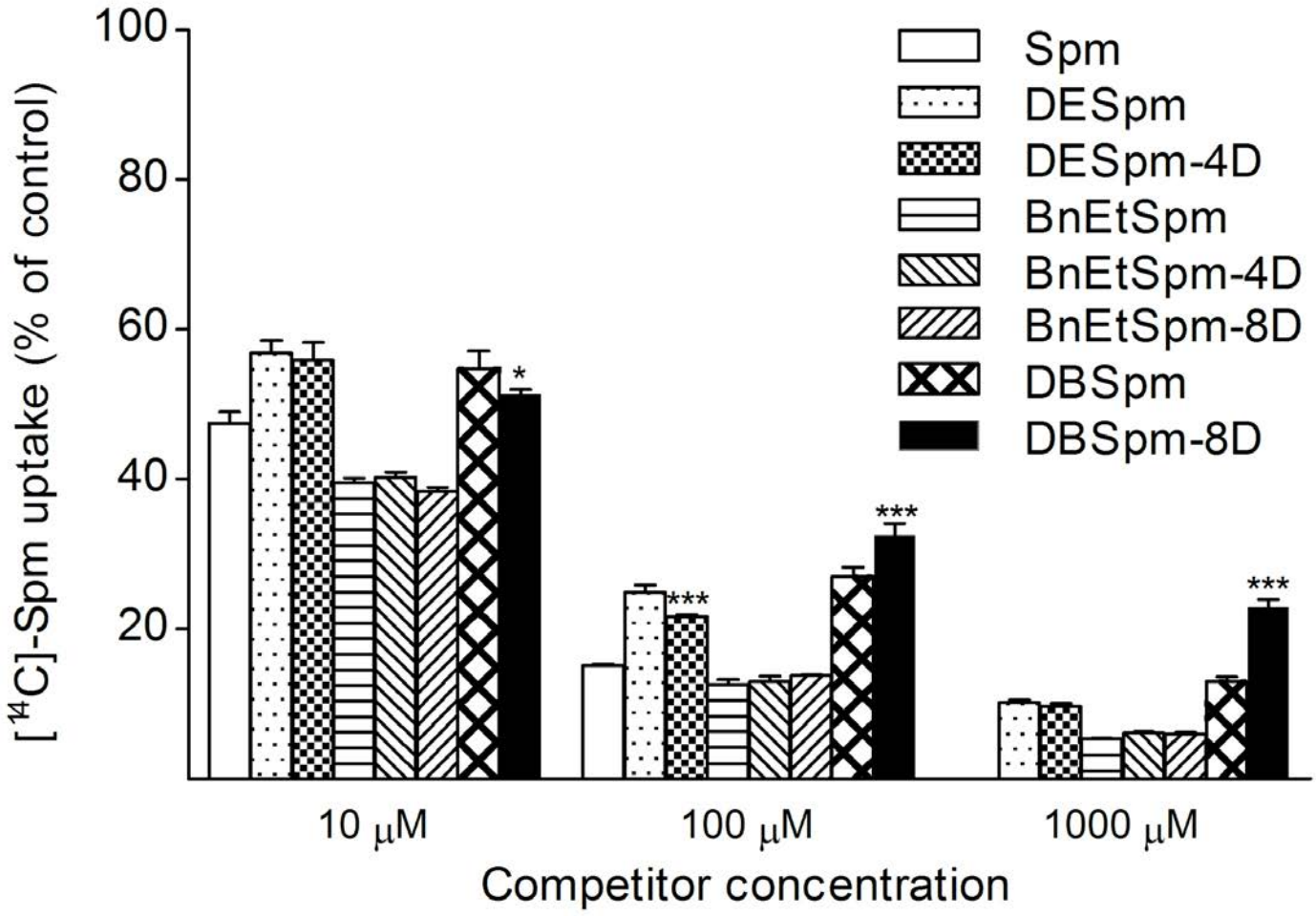
**DBSpm-4D (1)**

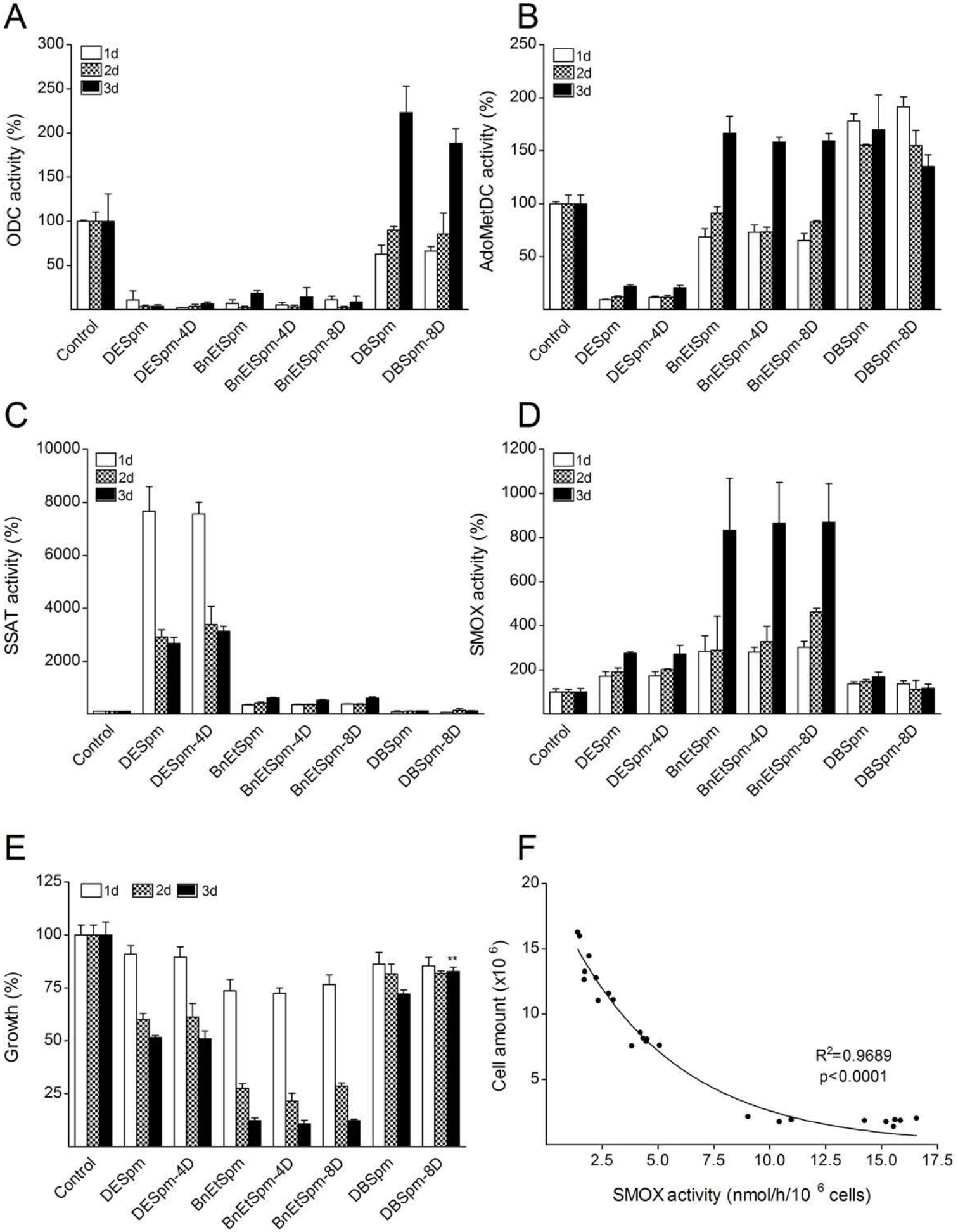


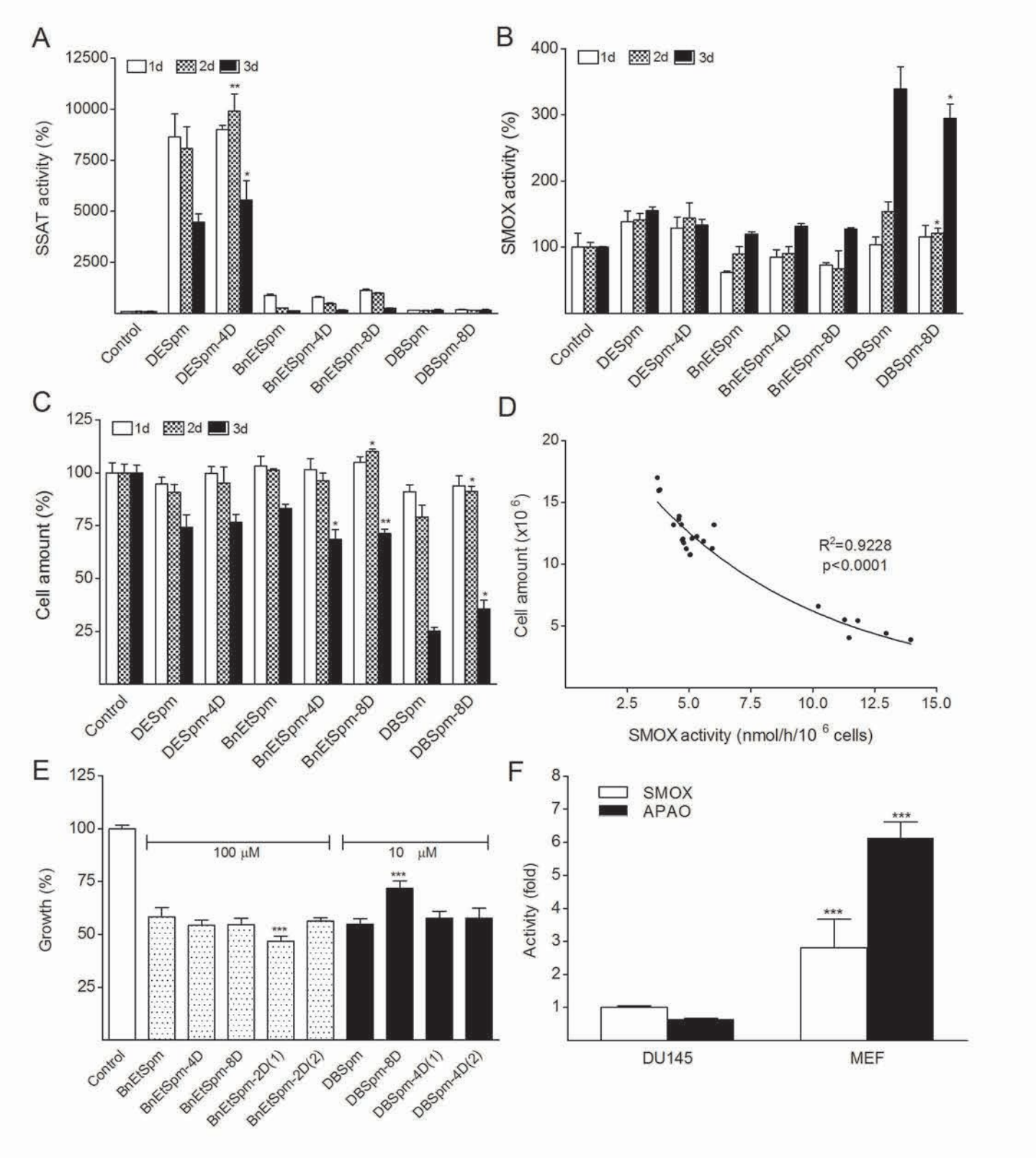
**Spm**

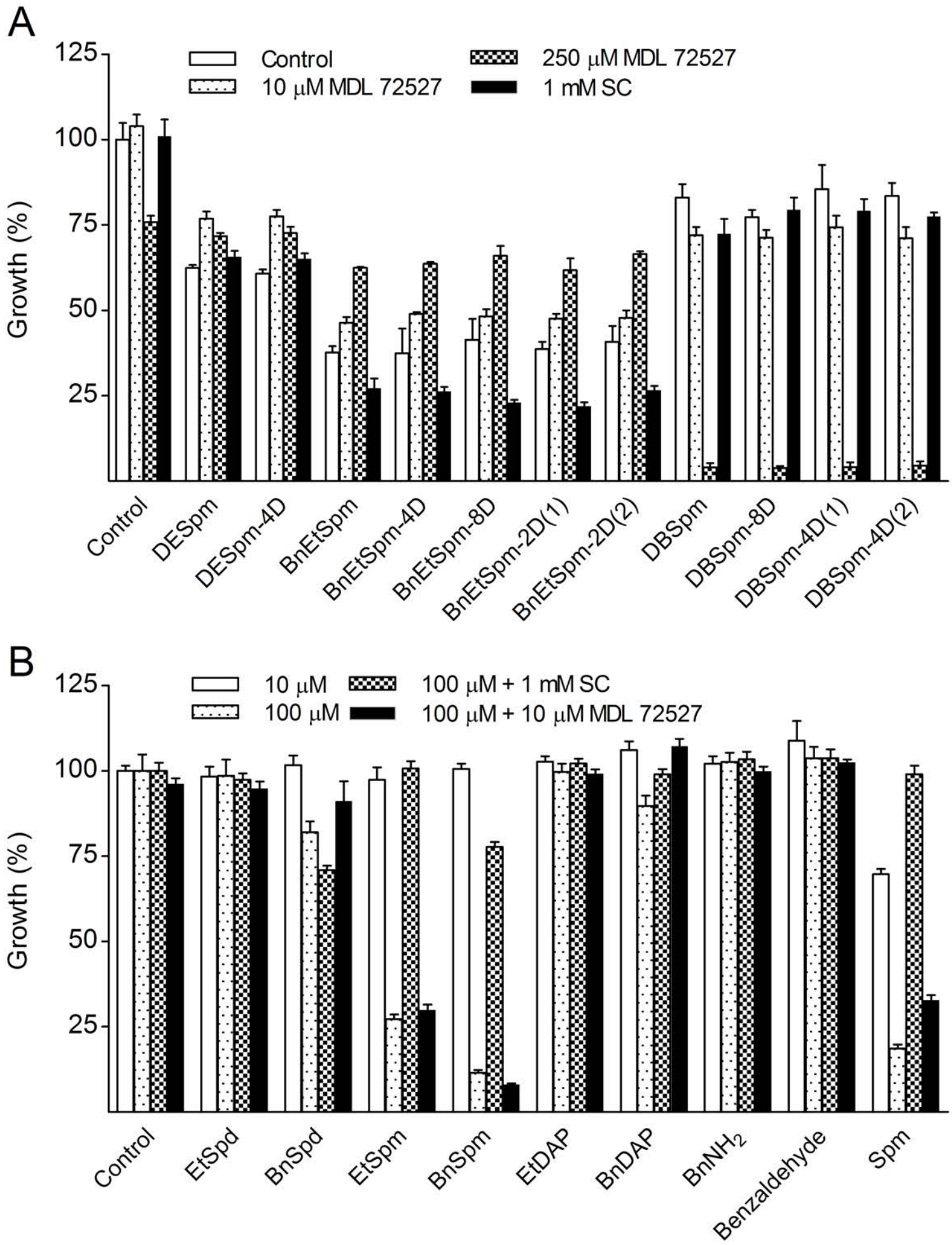
A
SMOX (% of Spm)
125
100
75
25
20
15
10
5
***
Spm
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm
DBSpm-8D
DBSpm-4D(1)
DBSpm-4D(2)
EtSpd
BnSpd
EtSpm
BnSpm
EtDAP
BnDAP
BnNH2
Put
Spd
N1-AcSpd
B
APAO (% of N1-AcSpd)
125
100
75
50
25
***
N1-AcSpd
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm
DBSpm-8D
DBSpm-4D(1)
DBSpm-4D(2)
EtSpd
BnSpd
EtSpm
BnSpm
EtDAP
BnDAP
BnNH2
Put
Spd
Spm
C
SSAO (% of Spm)
125
100
75
50
25
***
Spm
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm
DBSpm-8D
DBSpm-4D(1)
DBSpm-4D(2)
EtSpd
BnSpd
EtSpm
BnSpm
EtDAP
BnDAP
BnNH2
Put
Spd
N1-AcSpd


A
SSAO (% of Spm)
50 μM
500 μM
Put
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm
DBSpm-8D
DBSpm-4D(1)
DBSpm-4D(2)
EtSpd
BnSpd
EtSpm
EtDAP
B
DAO (% of Put)
Spd
Spm
DESpm
EBSpm
BBSpm
EtSpd
BnSpd
EtSpm
BnSpm
BnDAP
C
APAO (% of N1-AcSpd)
EtSpd
EtSpm
EtDAP
BnDAP
BnNH2
Put
Spd
Spm
D
SMOX (% of Spm)
EtSpd
BnSpd
EtDAP
BnDAP
BnNH2
Put
Spd
N1-AcSpd


100
80
60
40
20
[14C]-Spm uptake (% of control)
Spm
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
DBSpm
DBSpm-8D
*
***
***
***
10 μM
100 μM
1000 μM
Competitor concentration


A
ODC activity (%)
1d
2d
3d
Control
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
DBSpm
DBSpm-8D
B
AdoMetDC activity (%)
C
SSAT activity (%)
D
SMOX activity (%)
E
Growth (%)
**
F
Cell amount (x10 6)
R2=0.9689
p<0.0001
SMOX activity (nmol/h/10 6 cells)


A
SSAT activity (%)
12500
10000
7500
5000
2500
1d
2d
3d
Control
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
DBSpm
DBSpm-8D
B
SMOX activity (%)
400
300
200
100
C
Cell amount (%)
125
100
75
50
25
D
Cell amount (x10 6)
20
15
10
5
R2=0.9228
p<0.0001
2.5
5.0
7.5
10.0
12.5
15.0
SMOX activity (nmol/h/10 6 cells)
E
Growth (%)
100 μM
10 μM
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm-4D(1)
DBSpm-4D(2)
F
Activity (fold)
SMOX
APAO
DU145
MEF

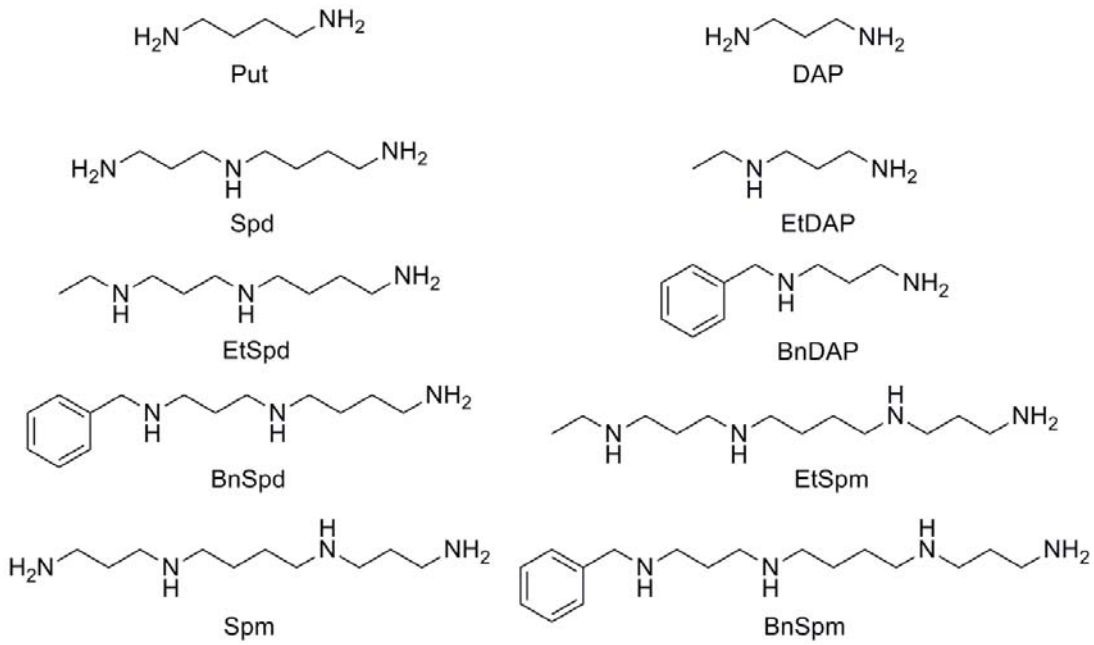

A
125
100
75
50
25
Growth (%)
Control
10 μM MDL 72527
250 μM MDL 72527
1 mM SC
Control
DESpm
DESpm-4D
BnEtSpm
BnEtSpm-4D
BnEtSpm-8D
BnEtSpm-2D(1)
BnEtSpm-2D(2)
DBSpm
DBSpm-8D
DBSpm-4D(1)
DBSpm-4D(2)
B
125
100
75
50
25
Growth (%)
10 μM
100 μM
100 μM + 1 mM SC
100 μM + 10 μM MDL 72527
Control
EtSpd
BnSpd
EtSpm
BnSpm
EtDAP
BnDAP
BnNH2
Benzaldehyde
Spm

**Supplementary information**

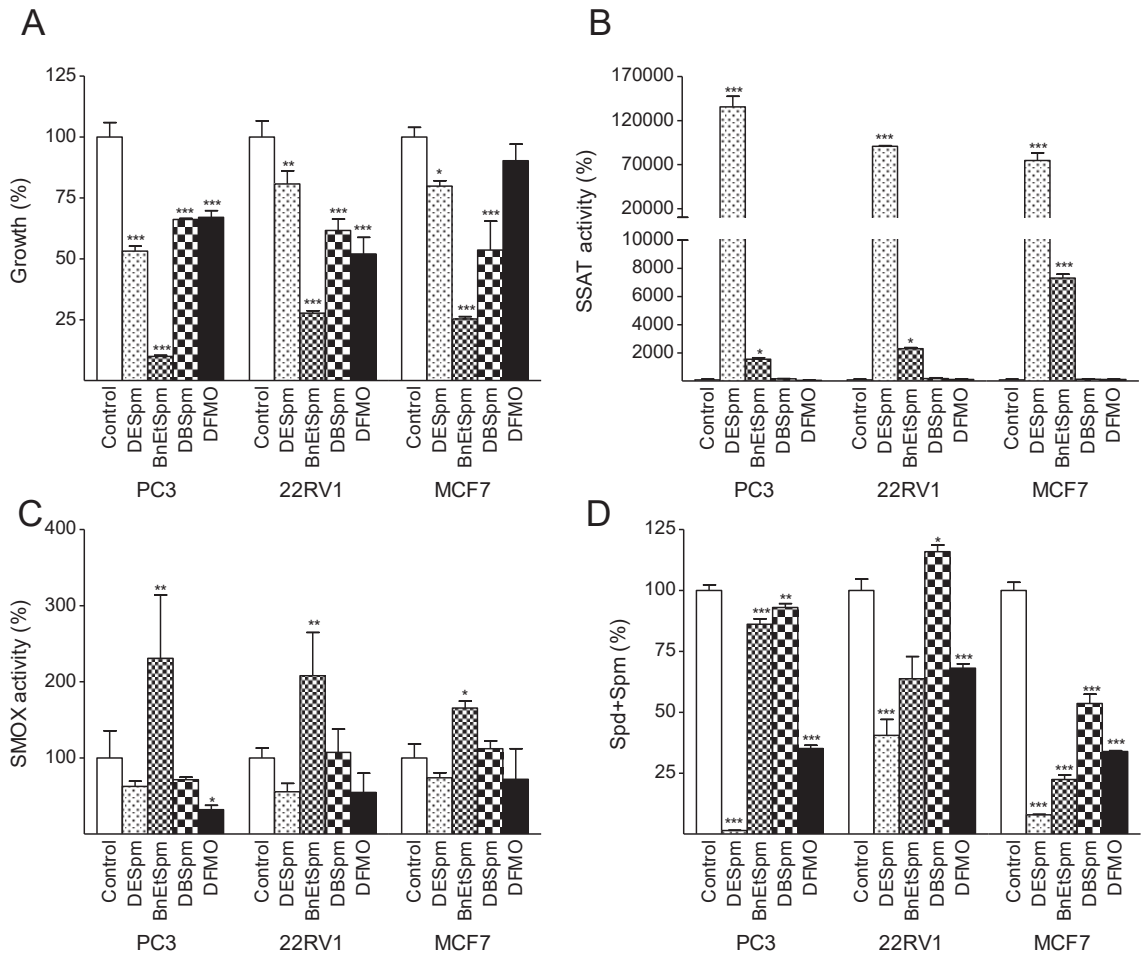
**Controlling of *N*-Alkylpolyamine Analog Catabolism by Selective Deuteration**

Sebahat Ucal, Merja R. Häkkinen, Aino-Liisa Alanne, Leena Alhonen, Jouko Vepsäläinen, Tuomo A. Keinänen and Mervi T. Hyvönen

School of Pharmacy, University of Eastern Finland, Biocenter Kuopio, Yliopistonranta 1B, 70210 Kuopio, Finland.




Supplementary Figure S1. Structures and abbreviations of the natural polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) and the metabolites of *N*-alkylated analogues used in the study. DAP, diaminopropane; EtDAP, $N^1$-ethyldiaminopropane; EtSpd, $N^1$-ethylspermidine; EtSpm, $N^1$-etylspermine; BnDAP, $N^1$-benzyldiaminopropane; BnSpd, $N^1$-benzylspermine; BnSpm, $N^1$-benzylspermine.




Supplementary Figure S2. Effect of parent analogues on the (A) growth, (B) SSAT activity, (C) SMOX activity, and (D) polyamine levels (Spd+Spm) in PC3 (prostate), 22RV1 (prostate) and MCF7 (breast) cancer cell lines. The cells were treated with 10 μM analogs or 5 mM DFMO for 3 days. Data are means ± S.D., n = 3. Statistical significance *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ as compared to control sample.