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METABOLISM OF N-ALKYLATED SPERMINE ANALOGUES BY POLYAMINE AND SPERMINE OXIDASES

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SUMMARY

N-alkylated polyamine analogues have potential as anticancer and antiparasitic drugs. However, their metabolism in the host has remained incompletely defined thus potentially limiting their utility. Here, we have studied the degradation of three different spermine analogues *N,N'*-bis-(3-ethylaminopropyl)butane-1,4-diamine (DESPM), *N*-(3-benzyl-aminopropyl)-*N'*-(3-ethylaminopropyl)butane-1,4-diamine (BnEtSPM) and *N,N'*-bis-(3-benzylaminopropyl)butane-1,4-diamine (DBSPM) and related mono-alkylated derivatives as substrates of recombinant human polyamine oxidase (APAO) and spermine oxidase (SMO). APAO and SMO metabolized DESPM to EtSPD ($K_{m(\text{APAO})}=10\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=1.1\text{s}^{-1}$ and $K_{m(\text{SMO})}=28\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.8\text{s}^{-1}$, respectively), metabolized BnEtSPM to EtSPD ($K_{m(\text{APAO})}=0.9\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=1.1\text{s}^{-1}$ and $K_{m(\text{SMO})}=51\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.4\text{s}^{-1}$, respectively), and metabolized DBSPM to BnSPD ($K_{m(\text{APAO})}=5.4\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=2.0\text{s}^{-1}$ and $K_{m(\text{SMO})}=33\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.3\text{s}^{-1}$, respectively). Interestingly, mono-alkylated spermine derivatives were metabolized by APAO and SMO to SPD (EtSPM $K_{m(\text{APAO})}=16\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=1.5\text{s}^{-1}$; $K_{m(\text{SMO})}=25\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=8.2\text{s}^{-1}$; BnSPM $K_{m(\text{APAO})}=6.0\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=2.8\text{s}^{-1}$; $K_{m(\text{SMO})}=19\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.8\text{s}^{-1}$, respectively). Surprisingly, EtSPD ($K_{m(\text{APAO})}=37\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=0.1\text{s}^{-1}$; $K_{m(\text{SMO})}=48\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.05\text{s}^{-1}$) and BnSPD ($K_{m(\text{APAO})}=2.5\mu\text{M}$, $k_{\text{cat}(\text{APAO})}=3.5\text{s}^{-1}$; $K_{m(\text{SMO})}=60\mu\text{M}$, $k_{\text{cat}(\text{SMO})}=0.54\text{s}^{-1}$) were metabolized to SPD by both the oxidases. Furthermore, we studied the degradation of DESPM, BnEtSPM or DBSPM in the DU145 prostate carcinoma cell line. The same major metabolites EtSPD and/or BnSPD were detected both in the culture medium and intracellularly after 48 hours of culture. Moreover, EtSPM and BnSPM were detected from cell samples. Present data shows that inducible SMO parallel with APAO could play an important role in polyamine based drug action, i.e. degradation of parent drug and its metabolites, having significant impact on efficiency of these drugs, and hence for the development of novel *N*-alkylated polyamine analogues.

Keywords

Polyamines; *N*-alkylated polyamine analogues; Flavin-dependent amino-oxidoreductases; Spermine oxidase; Polyamine oxidase

INTRODUCTION

The polyamines (PA) spermidine (SPD), spermine (SPM) and their diamine precursor putrescine (PUT) are small organic bases, which are ubiquitous in mammalian cells (Tabor and Tabor 1984). Their intracellular levels are strictly regulated by several enzymes and the cell membrane transport system (Seiler 2004). Dysregulated polyamine metabolism has been associated with neoplastic transformation and cancer cell growth (Pegg 1988; Pegg and Feith 2007). Parasitic diseases are an enormous health problem, especially in many developing countries, requiring cost effective drugs. Fortunately, the polyamine metabolic pathway has been found to contain several potential drug targets in parasites (Heby et al. 2007; Kaiser et al. 2006; Muller et al. 2008; Reguera et al. 2005). Thus, targeting differences in polyamine metabolism between host and parasite, or healthy and malignant tissue may offer a selective advantage and avoid significant host toxicity. Therefore, polyamine metabolism or their cellular targets offers a rational basis for drug design against both parasitic diseases and diseases caused by uncontrolled proliferation (Casero and Marton 2007; Heby et al. 2007).

Various polyamine analogues and polyamine synthesis inhibitors have been investigated as chemopreventive, chemotherapeutic or antiparasitic agents (Pegg et al. 1995; Seiler 2003; Williams 1997). It is known that relatively modest structural modifications in the PA backbone or incorporation of terminal *N*-alkyl substituent(s) may evoke significant differences in their chemical and biological behaviour (Bergeron et al. 1997; Byers et al. 1990). Some of the *N*-alkylated polyamine analogues have already shown promising results in various model systems of chemotherapy (Casero and Marton 2007; Seiler 2003). *N*-ethyl substituted polyamines, like diethylnorspermine (DENSPM) have displayed cytotoxic activity and are potential therapeutics for cancer, while *N*-benzyl substituted polyamines have shown to be effective in the treatment for malaria in a mouse model (Bitonti et al. 1989; Casero and Marton 2007; Edwards et al. 1991). Close structural PA analogues mimic their natural counterparts in regulatory functions, deplete natural polyamines in cells and most importantly block the compensatory uptake of polyamines (Bergeron et al. 1997). Unfortunately, most of the clinical trials have failed due to apparent toxicity and/or low efficacy of the studied compounds. One reason for low cell response to antitumor polyamine analogues may be the catabolism of the active drug by different oxidases (Lawson et al. 2002). Interestingly, at the same time, the biological activity of specific polyamine analogues has been shown to originate from the active catabolism of cellular polyamines by SMO to cytotoxic hydrogen peroxide (Pledgie et al. 2005).

APAO and SMO are two of the key enzymes of polyamine catabolism. APAO was characterized in 1977 when Hölttä purified rat liver APAO enzyme (Hölttä 1977). Since then, APAO has been purified from very few other sources and the human enzyme was cloned in 2003 (Libby and Porter 1987; Tsukada et al. 1988; Wu et al. 2003). SMO (PAOh1) was initially cloned and characterized in 2001 (Wang et al. 2001). Cloning of both the enzymes has made them readily available for recombinant protein production that has facilitated further testing of their substrate properties (Järvinen et al. 2006a; Wang et al. 2003; Wang et al. 2005a). During polyamine catabolism, SPM and SPD are first acetylated by spermidine/spermine *N*¹-acetyltransferase (SSAT) and subsequently oxidized by APAO to produce SPD and PUT, respectively, and to yield stoichiometric amounts of hydrogen

peroxide and 3-acetamidopropanal. APAO prefers acetylated polyamines over non-acetylated polyamines, and its natural substrates are N^1, N^{12} -DiAcSPM, N^1 -AcSPM and N^1 -AcSPD (Seiler 1995; Wang et al. 2005a). In addition to the natural polyamines, some terminally N -alkylated polyamines are degraded by APAO, and some of the otherwise metabolically stable polyamine analogues are catabolized when certain aldehydes are present in the reaction mixture (Järvinen et al. 2005; Järvinen et al. 2006a; Vujcic et al. 2003; Wu et al. 2003). By contrast, SMO possesses very different substrate specificities for the natural polyamines as compared with APAO. It prefers non-acetylated polyamines, efficiently oxidizing SPM, less efficiently N^1 -AcSPM, and does not use SPD as a substrate (Wang et al. 2003). In addition to APAO and SMO, also other oxidases (such as mono and diamine oxidases) can metabolize polyamines by selectively removing primary N -terminal amine, which will lead to subsequent spontaneous cleavage at the exo side of the N^4 -amine of SPM (Fig. 1.) (Lee and Sayre 1998; Seiler 2004; Agostinelli et al 2004).

Although APAO and SMO may significantly affect the efficacy of polyamine-based drugs, metabolic studies with purified enzymes and N -alkylated PA analogues have been very limited (Lawson et al. 2002; Wang et al. 2003; Wang et al. 2005a; Wu et al. 2003). A key for detailed metabolic studies has been the development of a novel accurate LC-MS/MS method for determining polyamines, N -alkylated analogues and their metabolites from biological samples. Using LC-MS/MS method we were able to verify the complicity of APAO-mediated degradation of DESPM (Häkkinen et al. 2007; Häkkinen et al. 2008).

In this study we have thoroughly investigated the substrate properties of N, N' -bis-(3-ethylaminopropyl)butane-1,4-diamine (DESPM), N -(3-benzyl-aminopropyl)- N' -(3-ethylaminopropyl)butane-1,4-diamine (BnEtSPM) and N, N' -bis-(3-benzylaminopropyl)butane-1,4-diamine (DBSPM) and their predicted secondary metabolites with APAO and SMO. In addition, experiments were performed to evaluate the cellular accumulation, catabolism, and excretion of the metabolites of N, N' -bis-alkylated spermine analogues in DU145 prostate cancer cells. Results of this study provide important information of the substrate properties of these two catabolic enzymes that will benefit future drug design.

MATERIALS AND METHODS

Reagents

Ultra-gradient HPLC-grade acetonitrile (ACN) was from J.T. Baker, heptafluorobutyric acid (HFBA, >99%) from Fluka, benzonitrile from Lancaster, and succinonitrile- d_4 and benzylamine from Aldrich. Formic acid, glycine and NaOH were from Sigma. Ultrapure water was prepared using a Milli-Q Gradient system (Millipore, Milford, MA, USA). Human recombinant APAO and SMO were produced as described earlier (Järvinen et al. 2005). APAO and SMO were stored frozen in 20% glycerol at -80°C , until use. Some loss of activity was detected during storage, thus 250 μM SPM or N^1 -AcSPD activity controls in triplicates were always included for all the analyses. Propane-1,3-diamine dihydrochloride (DAP, 98%) was from Aldrich. Butane-1,4-diamine dihydrochloride (PUT, 98%), N^1 -(3-aminopropyl)butane-1,4-diamine trihydrochloride (SPD, 98%), N, N' -bis-(3-aminopropyl)butane-1,4-diamine tetrahydrochloride (SPM, >95%) were from Sigma. All the deuterated reference compounds except those described below were prepared as previously described (Häkkinen et al. 2009). Benzylamine hydrochloride (BnNH_2) was prepared from benzylamine and recrystallized from ethanol-ethyl acetate.

α, α - $^2\text{H}_2$ -Benzylamine hydrochloride (BnNH_2 -2D) was prepared from benzonitrile (1 g, 9.7 mmol) and LiAlD_4 (815 mg, 19.4 mmol) using the previously described methods (Häkkinen et al. 2009) to give BnNH_2 -2D as colourless crystals. (974 mg, 69 %). ^1H NMR

(D₂O): δ 7.57-7.43 (5H, m); ¹³C NMR (D₂O): δ 135.3, 132.1 (4C), 131.7, 45.4 (quintet, J = 22 Hz).

1,1,2,2,3,3,4,4-²H₈-Butane-1,4-diamine dihydrochloride (PUT-8D) was prepared from succinonitrile-d₄ (200 mg, 2.38 mmol) by catalytic deuteration essentially as described in (Smith and Daves 1978) to give PUT-8D as colourless crystals. (158 mg, 39 %). ¹³C NMR (D₂O): δ 41.0 (2C, quintet, J = 22 Hz), 25.6 (2C, quintet, J = 19 Hz)

N¹-(3-Benzylamino-1,1,2,2-²H₄-propyl)butane-1,4-diamine trihydrochloride (BnSPD-4D) was prepared from N¹-benzyl-bis-N¹,N³-(2-nitrobenzenesulfonyl)-2,2,3,3-²H₄-propane-1,3-diamine as described in (Häkkinen et al. 2009) (808 mg, 1.5 mmol) and N-(4-iodobutyl)phthalimide (Järvinen et al. 2006b) (545 mg, 1.65 mmol) using method described in (Häkkinen et al. 2009) to give BnSPD-4D (193 mg, 37 %) as a colourless solid. ¹H NMR (D₂O): δ 7.55-7.48 (5H, m), 4.29 (2H, s), 3.20 (2H, s), 3.16-3.10 (2H, m), 3.10-3.03 (2H, m), 1.85-1.73 (4H, m); ¹³C NMR (D₂O): δ 133.3, 132.7 (2C), 132.6, 132.2 (2C), 54.1, 49.8, 46.6 (2C, s + m), 41.7, 26.7, 25.6, 24.7 (quintet, J = 20 Hz).

N,N'-bis-(3-Benzylaminopropyl)butane-1,4-diamine tetrahydrochloride (DBSPM) was prepared from N¹-benzyl-bis-N¹,N³-(2-nitro-benzenesulfonyl)propane-1,3-diamine (Häkkinen et al. 2009) (1 g, 1.87 mmol) and 1,4-diiodobutane (276 mg, 0.89 mmol) using methods described in (Häkkinen et al. 2009) to give DBSPM (211 mg, 45 %) as a colourless solid. ¹H NMR chemical shifts as described earlier (Bergeron et al. 2001); ¹³C NMR (D₂O): δ 133.4 (2C), 132.8 (4C), 132.7 (2C), 132.2 (4C), 54.1 (2C), 49.9 (2C), 47.4 (2C), 46.8 (2C), 25.6 (2C), 25.5 (2C).

N,N'-bis-(3-Benzylamino-1,1,2,2-²H₄-propyl)butane-1,4-diamine tetrahydrochloride (DBSPM-8D) was prepared from N¹-benzyl-bis-N¹,N³-(2-nitrobenzenesulfonyl)-2,2,3,3-²H₄-propane-1,3-diamine (Häkkinen et al. 2009) (1.13 g, 2.1 mmol) and 1,4-diiodobutane (310 mg, 1 mmol) using the methods described in (Häkkinen et al. 2009) to give DBSPD-8D (318 mg, 59 %) as a colourless solid. ¹H NMR (D₂O): δ 7.56-7.47 (10H, m), 4.29 (4H, s), 3.20 (4H, s), 3.17-3.08 (4H, m), 1.85-1.73 (4H, m); ¹³C NMR (D₂O): δ 133.3 (2C), 132.7 (4C), 132.6 (2C), 132.2 (4C), 54.1 (2C), 49.8 (2C), 46.6 (4C, s + m), 25.6 (2C), 24.7 (2C, quintet J = 19 Hz).

Instrumentation

LC separations, MS/MS detection and analysis of the compounds were achieved with Agilent 6410 Triple Quad LC/MS equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler. Data acquisition and analysis were performed using an Agilent MassHunter Workstation software (Agilent Corporation, MA, USA). ¹H and ¹³C NMR spectra were measured on a Bruker Avance (Bruker, Rheinstetter, Germany) 500 DRX spectrometer as described earlier (Häkkinen et al. 2009).

Analytical conditions

LC-MS/MS experiments were based on selected reaction monitoring (SRM) analysis and were performed essentially as described earlier (Häkkinen et al. 2008). The chromatographic separations were carried out using a Phenomenex Gemini reversed phase C18 column (3 μ m, 50 mm \times 2 mm, 110 Å) protected with a Phenomenex C18 guard column (4 mm \times 2 mm). A gradient solvent system consisting of 0.1% (v/v) HFBA in water (solvent A) and 0.1% (v/v) HFBA in ACN (solvent B) was used as before (Häkkinen et al. 2008), but the gradient was increased from 2–50% B over 12 min at a flow rate of 0.2 mL/min. Two time segments were used, turning point being 7.5 min. In the first time segment (0–7.5 min) dwell times were set to 100 (10 ion transitions), and after 7.5 min dwell time were 20 (30 ion transitions). The precursor and the product spectra of each polyamine were recorded

similarly as described earlier (Häkkinen et al. 2007). Fragmentor voltage value was set to 60 V for DAP, DAP-2D, PUT, PUT-8D, BnNH₂ and BnNH₂-2D, and to 90 V for rest of the analytes. Precursor ions, selected product ions for quantification and qualification, and collision energy values for all analytes used in the quantitative SRM analysis are given in Table 1. DAP and PUT have only one product ion.

Preparation of standards and quality controls

Standard working solutions (concentrations of 0.03, 0.1, 0.3, 1, 3, 10, 30 and 60 µM) and working solutions for quality control (QC) samples (concentrations of 0.05, 0.2, 2 and 20 µM) in 90 mM glycine-NaOH-formic acid buffer, and internal standard (IS) working solution containing 1 µM of each 14 deuterated amine in water, were prepared essentially as described earlier (Häkkinen et al. 2008).

Two types of calibration standards and QC samples were prepared using these working solutions by adding 50 µl of each STD working solution or QC working solution, 50 µl of 1 µM IS working solution and 25 µl 0.5 % HFBA (calibration curve 1 for enzyme kinetic analyses) or 10 % HFBA (calibration curve 2 for cell culture experiments). Additional HFBA was used to maintain the chromatographic performance of samples containing sulphosalicylic acid. Samples were transferred into Agilent polypropylene vial inserts for the LC-MS/MS analysis. The calibration curves included also a blank sample and a “zero” sample as described earlier (Häkkinen et al. 2008).

Calibration curves and assay validation

Calibration curves were constructed from accurate concentrations of working solutions of each analyte in x-axis, and peak-area ratio sample vs. internal standard in y-axis using 1/x weighted linear or quadratic (for SPD and EtSPM) least-squares regression model. Assay validation was performed according to the FDA guideline for bioanalytical method validation (FDA 2001) essentially as described earlier (Häkkinen et al. 2008).

Enzyme Kinetic Analyses with Human Recombinant APAO and SMO

The kinetic studies were carried out twice in triplicates at 4 to 7 different (SMO 25–250 µM and 100–1000 µM; APAO 10–1000 µM) substrate concentrations. The enzyme stock solution was diluted with 50 mM sodium phosphatebuffer (pH 8.0) (containing 0.1% Triton X-100) before kinetic studies to yield 0.1–2.0 µg/10 µl of APAO or SMO for each reaction mixture. Reactions were carried out in a total volume of 180 µl in the 100 mM glycine-NaOH at pH 9.5 buffer and were allowed to proceed for 2 to 60 min at 37 °C before addition of 20 µl of 50 % (v/v) formic acid. All kinetic determinations included a T_{1/2} reference (incubated half of the reaction time of actual samples to monitor linearity of reaction) and the reaction mixture without enzyme supplements both with the highest studied drug concentration treated identically as analysed sample to exclude non-enzymatic degradation of studied compounds. Moreover, with some selected reaction mixtures preincubation with 100 µM MDL 72,527 was used to inactivate SMO in order to rule out any nonenzymatic degradation of tested drug. LC-MS/MS was used to determine the concentrations of the polyamines and their analogues as described above. Prior to LC-MS/MS analysis, concentrated samples (> 50 µM) were diluted with 90 mM glycine-NaOH-formic acid buffer and all samples were passed through a 0.22 µm filter. LC-MS/MS samples were prepared similarly to calibration standards and QC samples (Häkkinen et al. 2008).

SMO activity at different pH in Bis-Tris propane buffer

Fixed 500 µM SPM in 170 mM Bis-Tris propane at pH 7.5, 8.0, 8.5, 9.0, 9.5 or 10.0 was used with 0.2 µg of SMO in total volume of 180 µl, incubated for 5 or 2.5 minutes at 37°C

water bath. Reactions carried out twice in triplicates were stopped by adding 20 μl of 50 % sulphosalicylic acid containing 100 μM 1,7-diaminoheptane and the SPD content was determined by using HPLC method described earlier (Hyvönen et al. 1992).

Cell culture

The prostate carcinoma cell line DU145 was obtained from American Type Culture Collection, USA. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum and 50 $\mu\text{g/ml}$ gentamycin under conditions of +37°C and 10 % CO_2 . The cells were harvested by trypsinization, counted electronically (Coulter Counter model Z1) and divided to six well plates. Cells were allowed to attach and after 24h, medium supplemented with or without 50 μM DESPM, BnEtSPM or BnSPM was added. Samples were collected at 24 and 48 hours post-treatment and analyzed for polyamines and *N*-alkylated analogues or their metabolites. LC-MS/MS samples were prepared similarly as calibration standards for calibration curve 2, and QC samples. Assays were carried out twice in triplicates.

Statistical Analyses

The data are expressed as the mean \pm S.D. A software package, GraphPad Prism 4.03 (GraphPad Software, Inc., San Diego, CA) was used for the analyses of enzyme kinetic data using Michaelis-Menten equation with nonlinear fitting (M_r of 55,382 for APAO and M_r of 68,000 for SMO were used in calculations).

RESULTS

LC-MS/MS method used for polyamine and polyamine analogue quantification

Polyamine quantification was based on the previously described LC-MS/MS method (Häkkinen et al. 2007; Häkkinen et al. 2008). Structures and abbreviations for the studied polyamines are shown in Table 2 together with their deuterium-labelled derivatives used in the LC-MS/MS measurements as the internal standards (Häkkinen et al. 2009). All of the 14 amines tested were quantifiable in a single run (Fig. 2). The dynamic range for each analyte was 0.03–60 μM , except for DAP which was 0.03–10 μM . The correlation coefficients (R^2) were always > 0.995 for all analytes. Inter-day accuracy of the assay for all analytes ranged between 88.8 and 109.8 % and the inter-day precision of the method was always < 13.4 %.

SMO activity at various pH

SMO activity was the highest between pH 8.5 and 9.5 (Fig. 3). Therefore, all the kinetic determinations were carried out at pH 9.5 being near optimal for both APAO and SMO (Hölttä 1977; Royo and Fitzpatrick 2005).

Terminally *N,N'*-bis-alkylated polyamine analogues as substrates for recombinant human APAO and SMO

As shown in Table 3, DESPM was catabolized to EtSPD by SMO and APAO. Minor *N*⁴-endo cleavage pathways resulting in formation of EtDAP was detected with both oxidases. Moreover, very minor de-ethylation takes place with APAO. BnEtSPM was likewise catabolized to EtSPD. However, in the case of SMO some competing debenzoylation occurred and evolved EtSPM was readily being cleaved to SPD. Moreover, some other cleavage site products were detected that were formed from secondary metabolites by both the oxidases. DBSPM was mainly metabolized to BnSPD by APAO. However, DBSPM was metabolized to BnSPM and BnSPD by SMO (Table 3).

Metabolism of predicted secondary metabolites by APAO and SMO

As shown in Table 3, EtSPM was readily degraded to SPD by both oxidases. APAO metabolized EtSPM variably, other competing pathways being less efficient. BnSPM was readily metabolized to SPD by APAO and SMO. APAO showed again more versatile substrate usage in comparison with SMO. Benzylamine is formed either from direct *N*¹-endo or *N*⁴-exo cleavage and subsequent chemical decomposition of benzylaminopropanol under alkaline conditions (Fig. 1). BnSPD was debenzylated to yield SPD by both oxidases. EtSPD was catabolized to SPD inefficiently by both the oxidases. Interestingly, EtDAP was not metabolized by either of the oxidases, but APAO was able to debenzylate BnDAP to DAP with reasonable catalytic efficiency (Table 3).

Metabolism of N,N'-bis-alkylated polyamine analogues in DU145 cells

The effects of 50 μ M analogues on intracellular polyamine levels in DU145 cells are shown in Table 4. DESPM accumulated readily inside the cells and most efficiently depleted the natural polyamine pools. EtSPD was detected as the major metabolite in conjunction with trace of EtSPM. BnEtSPM accumulated as efficiently as DESPM inside the cells, but did not deplete polyamine pools as efficiently as the treatment with DESPM did. EtSPD and EtSPM were detected as the major metabolites and some BnSPD accumulated during 48 hours in the BnEtSPM-treated cells. DBSPM was taken up least efficiently among the studied analogues. Moreover, it distorted intracellular polyamine pools less efficiently in comparison with DESPM and BnEtSPM. BnSPM and BnSPD were detected as the major metabolites from DBSPM. In culture medium from cell samples treated with DESPM or BnEtSPM, only EtSPD was detected together with intact drugs (data not shown). BnSPD and a trace amount of BnDAP (48 hour time point) were detected as the metabolites of DBSPM treatment. Total excretion of each metabolite from the cells was less than 0.2 μ M (equals to 400 pmol/metabolite in two ml of medium) in culture medium within 48 hours with all the drugs studied. Some PUT and SPD, at less than 0.8 μ M concentrations, were detected in control and drug supplemented culture media in 24 hour and 48 hour samples (2 ml medium/well, data not shown). Interestingly, no SPM was detected in any of the culture media samples (data not shown). Moreover, SPD was present at 2.5–5 times higher concentrations in drug-treated medium samples in comparison with control medium samples (data not shown). This could imply that SPM is metabolized to SPD prior to being excreted from the cells.

DISCUSSION

N-Alkylated PA-analogues have been shown to be metabolized by cellular oxidase(s) which are sensitive to MDL 72,527 (Bergeron et al. 1995; Bergeron et al. 1997; Bitonti et al. 1990; Bolkenius and Seiler 1989). The oxidase responsible was thought to be APAO but recent cloning and characterization of SMO has complicated the picture of how these drugs are being metabolized *in vivo* and *in vitro*. It has been clearly shown that human and murine recombinant APAO is capable of metabolizing DENSPM (Häkkinen et al. 2008; Vujcic et al. 2003; Wu et al. 2003). Some unsymmetrically *N*-substituted norspermine analogues are substrates of APAO (Wang et al. 2005a). In the case of SMO, studies have shown the affinity of many of the studied analogues to the enzyme and many of them have been thought to be inhibitors, not substrates (Vujcic et al. 2002; Wang et al. 2003). The assay method used has been based on fluorometric detection of hydrogen peroxide in a dual enzyme assay system (Wang et al. 2003). The fluorometric detection method is sensitive, but lacks the ability to distinguish which drug in a combination of analogues/polyamine is used as a substrate and how it is being metabolized in the reaction mixtures. However, other methods are valid for testing substrate properties of tested compounds and can be used in inhibition studies. A recent application of using ¹³C NMR for the detection of oxidase-

mediated catabolism of labelled tracer drug *in vitro* offers means to characterize different reaction products (Bacchi et al. 2009). NMR is a feasible method but lacks sensitivity, and mixtures of polyamine analogues and reaction products are sometimes hard to interpret accordingly. We have recently developed a novel LC-MS/MS method that was exploited to reveal versatile cleavage of DESPM by APAO and shed some light on earlier controversy related to degradation pathways of diethylated SPM analogues (Häkkinen et al. 2007; Häkkinen et al. 2008). Now, we have further extended these studies to analyze the metabolism of three different SPM analogues *in vitro* and *in situ*.

The LC-MS/MS method proved to be functional for analyzes of *N*-alkylated polyamine analogues and their metabolites in *in vitro* enzyme assay mixtures. Moreover, the method was sufficiently sensitive for quantifying polyamines and their *N*-alkylated analogues in cell and medium samples i.e. different sample matrix. The validity of the assay is based on the use of deuterated reference compounds with LC-MS/MS analysis of each compound. Advantages over existing HPLC methods are the increased sensitivity, and exact quantification and identification of each analyte (Häkkinen et al. 2008).

APAO- and SMO-mediated catabolism of *N*-alkylated polyamine analogues was mediated through the same major catabolic pathways. Both enzymes showed ability for versatile cleavages of the studied substrates in the pilot studies with DESPM, BnEtSPM or DBSPM. Thus, a systemic study with all predicted metabolites of DESPM, BnEtSPM or DBSPM was required to dissociate direct metabolism from the metabolism of secondary metabolites. SMO and APAO metabolized DESPM to EtSPD, thus mimicking the degradation of N^1, N^{12} -DiAcSPM by APAO. The aromatic benzyl end of BnEtSPM was strongly preferred by SMO and APAO, resulting in formation of EtSPD. However, with SMO there was an active debenzilation pathway present, and evolved EtSPM was effectively further metabolized to SPD. In the case of SMO, N^1 -exo and N^4 -exo cleavage were equally active when DBSPM was used as a substrate. Furthermore, some BnDAP was detected indicating the presence of N^4 -endo cleavage. APAO strongly preferred N^4 -exo cleavage yielding BnSPD with DBSPM as substrate and other competing pathways were less than 5 % active when compared to the catalytic-centre activity of the major pathway.

EtSPM was metabolized very efficiently to SPD by SMO. It has been shown that SMO is able to degrade N^1 -AcSPM and it seems that ethyl substituent that retains the amine as positively charged strongly accelerates reaction velocity (Wang et al. 2003). Similarly, SPD was a major metabolite with APAO, but some other competing pathways were also detected. Interestingly, with charge retaining EtSPM and DESPM the reaction velocity is slower when compared with N^1 -AcSPM and N^1, N^{12} -DiAcSPM, respectively (Järvinen et al. 2006a). BnSPM was metabolized to SPD by both oxidases, APAO being more efficient than SMO. Some other competing pathways were also present. EtSPD was a very weak substrate for both oxidases and was de-ethylated to SPD. However, BnSPD was very efficiently debenzylated by APAO to yield SPD, and with a 15 % reaction velocity by SMO in comparison to APAO. Moreover, APAO was capable in debenzylating BnDAP to DAP with a reasonable catalytic efficiency. SPD has been shown to be an inhibitor for SMO, but it seems that the aromatic *N*-terminal substituent evokes catalytic activity with SMO.

The metabolite formation was linear with all tested metabolites except for BnNH₂, which may also be formed via further chemical degradation of produced benzylaminopropanal liberating acrolein in the incubation solutions of high pH. This was noted as an increased reaction velocity of the formation of BnNH₂ after a longer (2x) incubation time in comparison to velocities obtained with T_{1/2} reference samples. Thus, rapid acidification of reaction mixture stabilizes benzylaminopropanal and prevents its chemical decomposition to BnNH₂ and acrolein. The previous data rules out N^1 -endo cleavage as a source of BnNH₂.

APAO is constitutively expressed in various tissues but SMO activity has been shown to be inducible by some of the *N*-alkylated polyamine analogues in certain cell lines (Wang et al. 2001; Devereux et al. 2003; Wang et al. 2005b). Significant APAO activity has been determined from post mortem human specimens (Suzuki et al. 1984) and furthermore SMO and APAO expression could be estimated by using virtual Northern analysis of EST databases as described in (Vujcic et al. 2003). Evaluation of the previous data strongly supports our view that the role of *N*-alkylated analogue degradation in determining the drug effect should be systematically studied. The basal activity of 431 ± 92 pmol/mg/h for SMO in DU145 cell has been determined earlier (Hyvönen et al. 2007). We did not determine the induction of SMO or APAO activity by *N*-alkylated analogues in our current study. Cell culture study was included to test the applicability of LC-MS/MS method for quantification of PA, *N*-alkylated PA analogues and their metabolites from different sample matrix than *in vitro* enzyme assay mixture.

Recently, SMO was shown to be responsible for drug-induced apoptosis acting as the primary source of hydrogen peroxide, and thus, playing an important role in polyamine catabolism-mediated cytotoxic response (Pledge et al. 2005). Our present data clearly shows that inducible SMO is capable of metabolizing *N*-alkylated polyamine analogues. This is not in agreement with earlier publications showing that none of the studied *N,N'*-bis-alkylated PA analogues were metabolized by SMO (Vujcic et al. 2002; Wang et al. 2003). Discrepancy could be explained by structural difference (3-3-3) or (4-4-4), instead of (3-4-3) carbon backbone in conjunction with different conditions in *in vitro* assay system. We did use the same cDNA described in (Vujcic et al. 2002) for production of recombinant SMO. However, Vujcic et al. did not purify enzyme instead they used cell lysates that could have caused DESPM behave like an inhibitor of SMO, not substrate like EtSPM. Thus, some apparent toxicity or low efficacies of the polyamine-based drugs in some cell lines may be explained by the ability of these two enzymes metabolize these drugs and their metabolites. It should be noted that some of the metabolites generated from *N*-alkylated PA analogues by SMO or APAO could be substrates for other cellular oxidases e.g. mono- or diamine oxidase. Although cellular APAO and SMO activities might have a significant impact on drug efficacy, kinetic studies of DESPM, BnEtSPM or DBSPM and their predicted secondary metabolites using purified polyamine oxidases have not been done until now. It is clear that present data should be taken into a consideration when novel *N*-alkylated polyamine analogues are being developed and used for the treatment of proliferative disorders or parasitic diseases. Further studies are clearly needed in order to assess the role of APAO and SMO in polyamine analogue-mediated drug response. Large versatility in their substrate properties and flexible cleavage of their substrates could implicate that SMO and APAO may have other natural substrates in addition to natural polyamines (Bacchi et al. 2009; Lentini et al. 2007). It should be noted that the studied analogues resembled SPM (3-4-3) carbon backbone. Many analogues based on norspermine (3-3-3) or homospermine (4-4-4) carbon backbone and several analogues having a variable length carbon chain between charged amines have been synthesized (Bergeron et al. 1997; Casero and Marton 2007). Alterations in carbon backbone could have a profound effect on their substrate properties with APAO and SMO and should be systematically studied in future. This work may require synthesis of appropriate reference compounds for exact quantification of reaction products by using LC-MS/MS. However, for screening of reaction mixtures just to detect any of the metabolites derived from the tested drugs could be carried out without internal reference compounds.

In summary, here we show for the first time that both APAO and SMO are capable of metabolizing several *N*-alkylated polyamine derivatives. This suggests that they might have a role in drug-mediated cytotoxic response, especially after the natural polyamines have been depleted. Although, exact mechanism of drug action of *N*-alkylated PA-analogues

remains obscure, polyamine metabolism is still an attractive target for drug design. It is evident that further studies including substrate properties on analogues with APAO and SMO are required.

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The abbreviations and trivial names

ACN	acetonitrile
APAO	(acetyl)polyamine oxidase (exo-N4-amino) [EC 1.5.3.11]
BnDAP	<i>N</i> ¹ -Benzyl-propane-1,3-diamine
BnDAP-4D	<i>N</i> ¹ -Benzyl-2,2,3,3- ² H ₄ -propane-1,3-diamine
BnEtSPM	<i>N</i> -(3-benzylaminopropyl)- <i>N'</i> -(3-ethylaminopropyl)butane-1,4-diamine
BnEtSPM-8D	<i>N</i> -(3-Benzylamino-1,1,2,2- ² H ₄ -propyl)- <i>N'</i> -(3-ethylamino-1,1,2,2- ² H ₄ -propyl)butane-1,4-diamine
BnNH₂	Benzylamine
BnNH₂-2D	α,α - ² H ₂ -Benzylamine
BnSPD	<i>N</i> ¹ -(3-Benzylaminopropyl)butane-1,4-diamine
BnSPD-4D	<i>N</i> ¹ -(3-Benzylamino-1,1,2,2- ² H ₄ -propyl)butane-1,4-diamine
BnSPM	<i>N</i> -(3-Aminopropyl)- <i>N'</i> -(3-Benzylaminopropyl)butane-1,4-diamine
BnSPM-4D	<i>N</i> -(3-Aminopropyl)- <i>N'</i> -(3-Benzylamino-1,1,2,2- ² H ₄ -propyl)butane-1,4-diamine
CID	collision energy
DAP	Propane-1,3-diamine
DAP-2D	1,1- ² H ₂ -Propane-1,3-diamine
DBSPM	<i>N,N'</i> -bis-(3-Benzylaminopropyl)butane-1,4-diamine
DBSPM-8D	<i>N,N'</i> -bis-(3-Benzylamino-1,1,2,2- ² H ₄ -propyl)butane-1,4-diamine
DENSPM	diethylnorspermine, <i>N,N'</i> -bis-(3-Ethylaminopropyl)-propane-1,3-diamine
DESPM	<i>N,N'</i> -bis-(3-ethylaminopropyl)butane-1,4-diamine
DESPM-4D	<i>N,N'</i> -bis-(3-Ethylamino-1,1- ² H ₂ -propyl)butane-1,4-diamine
EtDAP	<i>N</i> ¹ -Ethylpropane-1,3-diamine
EtDAP-2D	<i>N</i> ¹ -Ethyl-3,3- ² H ₂ -propane-1,3-diamine
EtSPD	<i>N</i> ¹ -(3-Ethylaminopropyl)butane-1,4-diamine trihydrochloride
EtSPD-2D	<i>N</i> ¹ -(3-Ethylamino-1,1- ² H ₂ -propyl)butane-1,4-diamine
EtSPM	<i>N</i> -(3-Aminopropyl)- <i>N'</i> -(3-ethylaminopropyl)butane-1,4-diamine

EtSPM-2D	<i>N</i> -(3-Aminopropyl)- <i>N'</i> -(3-ethylamino-1,1- ² H ₂ -propyl)butane-1,4-diamine
FDA	U.S. Food and Drug Administration, U.S.A
HFBA	heptafluorobutyric acid
HPLC	high pressure liquid chromatography
IS	internal standard
LC-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
MDL 72	527, <i>N,N'</i> -bis-(2,3-butadienyl)-1,4-butanediamine
NMR	nuclear magnetic resonance
<i>N</i>¹-AcSPD	<i>N</i> ¹ , <i>N</i> ¹² -Diacetylspermine
<i>N</i>¹-AcSPM	<i>N</i> ¹ , <i>N</i> ¹² -Diacetylspermidine
<i>N</i>¹	<i>N</i> ¹² -DiAcSPM, <i>N</i> ¹ , <i>N</i> ¹² -Diacetylspermine
PA	polyamine
PUT	putrescine, Butane-1,4-diamine
PUT-8D	1,1,2,2,3,3,4,4- ² H ₈ -Butane-1,4-diamine
SMO	(PAOh1), spermine oxidase [EC 1.5.3.-]
SPD	spermidine, <i>N</i> ¹ -(3-aminopropyl)butane-1,4-diamine
SPD-2D	<i>N</i> ¹ -(3-Amino-1,1- ² H ₂ -propyl)butane-1,4-diamine
SPM	spermine, <i>N,N'</i> -bis-(3-aminopropyl)butane-1,4-diamine
SPM-4D	<i>N</i> -(3-Amino-1,1,2,2- ² H ₄ -propyl)- <i>N'</i> -(3-aminopropyl)butane-1,4-diamine
SRM	selected reaction monitoring
SSAT	spermidine/spermine <i>N</i> ¹ -acetyltransferase [EC 2.3.1.57]
STD	standard
QC	quality control
QL	qualifier ion
QT	quantifier ion

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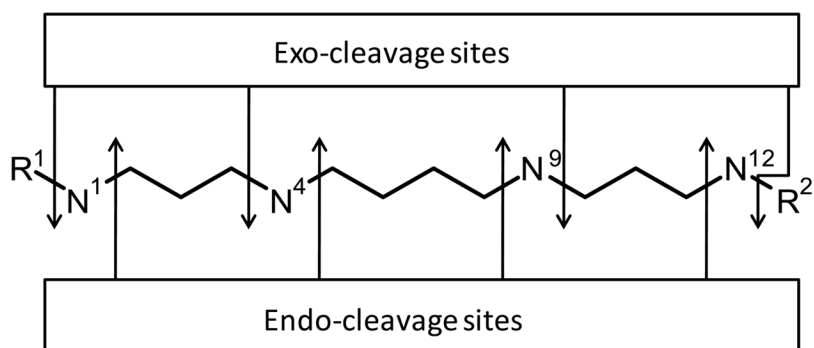


Fig 1. Endo- and exo-cleavage sites of *N*-alkylated PA analogue are shown with arrows. R¹, R² = Et and/or Bn.

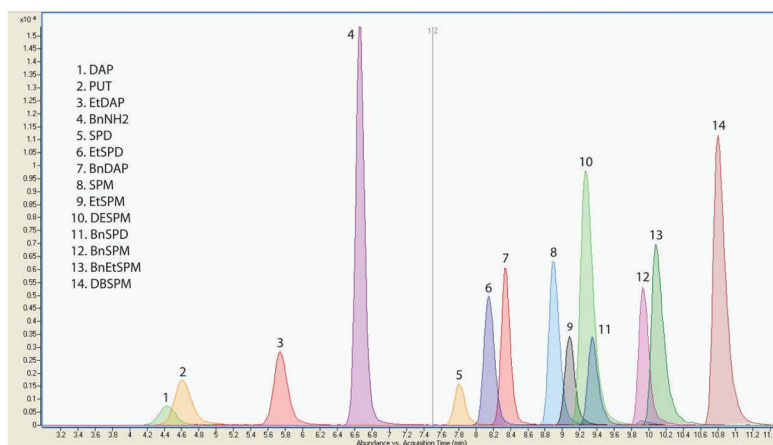


Fig. 2. Overlaid RP-LC-MS/MS SRM chromatograms of all the tested 14 amines, which were able to quantify in a single run. Interday accuracy for all was between 88.8–109.8 % and precision < 13.4 %.

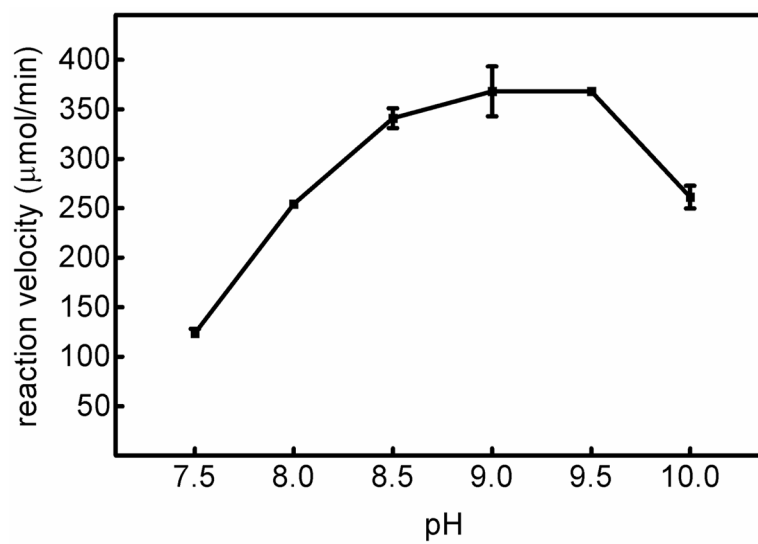


Fig. 3. Reaction velocities ($\mu\text{mol}/\text{min}$) for SMO were determined by using 500 μM SPM as a substrate at different pH in 170 mM Bis-Tris propane buffer.

Table 1

LC-MS/MS properties of polyamines used in this study

Amine standard	Precursor [M+H] ⁺	QT	CID (eV)	QL	CID (eV)	Deuterated internal standard	Precursor [M+H] ⁺	QT	CID (eV)	Retention time
DAP	75	58	5	-	-	DAP-2D	77	60	5	4.4
PUT	89	72	5	-	-	PUT-8D	97	80	5	4.6
EtDAP	103	86	5	58	15	EtDAP-2D	105	88	5	5.7
BnNH2	108	91	10	65	20	BnNH2-2D	110	93	10	6.7
SPD	146	72	15	112	10	SPD-2D	148	114	10	7.8
EtSPD	174	72	15	86	15	EtSPD-2D	176	88	15	8.2
BnDAP	165	148	5	91	20	BnDAP-4D	169	152	5	8.3
SPM	203	129	5	112	15	SPM-4D	207	133	5	8.9
EtSPM	231	157	10	129	10	EtSPM-2D	233	159	10	9.1
DESPM	259	157	10	112	20	DESPM-4D	263	159	10	9.3
BnSPD	236	148	15	112	15	BnSPD-4D	240	152	15	9.4
BnSPM	293	219	10	112	20	BnSPM-4D	297	223	10	9.9
BnEtSPM	321	219	10	112	20	BnEtSPM-8D	329	223	10	10.1
DBSPM	383	219	15	112	20	DBSPM-8D	391	223	15	10.8

Chemical structures with abbreviated names are shown in Table 2, and the chemical names are given in "Materials and Methods". QT, quantifier ion; QL, qualifier ion; CID, collision energy

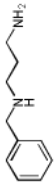

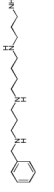

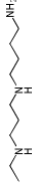
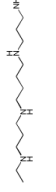

Table 2

Structures of the amine compounds in the study and their deuterated analogues as internal standards in LC-MS/MS quantification.

	Structure and abbreviation	Internal standard (IS)
DAP		DAP-2D
PUT		PUT-8D
SPD		SPD-2D
SPM		SPM-4D
EtDAP		EtDAP-2D
EtSPD		EtSPD-2D
EtSPM		EtSPM-2D
DESPM		DESPM-4D
BnNH ₂		BnNH ₂ -2D
BnDAP		BnDAP-4D
BnSPD		BnSPD-4D
BnSPM		BnSPM-4D
BnEtSPM		BnEtSPM-8D
DBSPM		DBSPM-8D

Table 3

Kinetic values of *N*-alkylated polyamine analogues and their predicted secondary metabolites for recombinant polyamine and spermine oxidases

Substrate	Metabolites	APAO			SMO		
		V_{max}	K_m	k_{cat}	V_{max}	K_m	k_{cat}
	DAP	36.9	84	0.62	NA	NA	NA
	PUT	18.5	0.4	0.31	0.33	54	0.01
	SPD	209	2.5	3.49	32.2	60	0.54
	BnDAP	NA	NA	NA	0.30	75	0.01
	BnNH ₂	26.1	5.0	0.44	13.2	9.8	0.22
	SPD	166	6.0	2.77	44.9	19	0.75
	SPM	3.79	12	0.06	NA	NA	NA
	BnSPD	7.51	20	0.12	NA	NA	NA
	BnNH ₂	5.59	1.4	0.09	9.79	8.3	0.16
	BnDAP	3.17	6.3	0.05	4.53	34	0.08
	BnSPD	121	5.4	2.02	17.8	33	0.30
	BnSPM	5.45	17	0.09	16.7	117	0.28
	PUT	0.38	36	0.01	0.28	47	0.00
	SPD	5.90	37	0.01	2.83	48	0.05
	EtDAP	0.23	48	0.00	0.09	79	0.00
	SPD	87.6	16	1.46	490	25	8.17
	SPM	1.73	11	0.03	NA	NA	NA
	EtSPD	2.51	25	0.04	NA	NA	NA
	EtDAP	1.48	4.7	0.02	NA	NA	NA
	EtSPD	64.2	10	1.07	45.6	28	0.76
	EtDAP	4.05	6.6	0.07	1.56	33	0.03
	EtSPM	4.66	17	0.08	NA	NA	NA
	EtSPM	4.17	1.9	0.07	8.28	651	0.14

Substrate	Metabolites	APAO			SMO		
		V _{max}	K _m	k _{cat}	V _{max}	K _m	k _{cat}
	EtSPD	63.5	0.9	1.06	21.1	51	0.35
	EtDAP	5.00	0.2	0.08	1.73	27	0.03
	BnNH ₂	5.96	1.0	0.09	8.89	49	0.15
	SPD	NA	NA	NA	8.44	5.7	0.14
	BnSPD	NA	NA	NA	4.95	34	0.08
	BnDAP	NA	NA	NA	1.51	28	0.02

Kinetic data was obtained as described in "Materials and Methods" by using human recombinant PAO or SMO. The abbreviations and structures are shown in Table 2. Apparent K_m(μ M), V_{max} (μ mol/min/ μ mol protein), k_{cat} (1/s). NA = not applicable. Reference activity for SMO by using 250 μ M SPM as a substrate was 460 \pm 62 μ mol/min and for APAO 270 \pm 15 μ mol/min by using 250 μ M N¹-AcSPD as a substrate.

Table 4

Intracellular polyamine and analogue levels in DU145 cells.

	cell number $\times 10^6$	PUT	SPD	SPM	Analogue	EtSPM	BnSPM	EtSPD	BnSPD
					(pmol/10 ⁶ cells)				
Control 0h	0.63 \pm 0.03	396 \pm 65	2409 \pm 320	1916 \pm 138	NA	NA	NA	NA	NA
Control 24h	1.06 \pm 0.08	246 \pm 16	2194 \pm 68	2226 \pm 72	NA	NA	NA	NA	NA
Control 48h	2.29 \pm 0.11	117 \pm 18	1626 \pm 224	1759 \pm 140	NA	NA	NA	NA	NA
DESPM 24h	1.01 \pm 0.02	ND	179 \pm 12	488 \pm 30	3799 \pm 270	ND	NA	31 \pm 3	NA
DESPM 48h	1.50 \pm 0.06	ND	35 \pm 4	111 \pm 14	3706 \pm 351	3.7 \pm 0.3	NA	41 \pm 6	NA
BnEtSPM 24h	0.97 \pm 0.08	ND	430 \pm 41	1543 \pm 74	3794 \pm 202	18 \pm 1	ND	34 \pm 3	ND
BnEtSpm 48h	1.72 \pm 0.08	ND	282 \pm 19	777 \pm 72	3929 \pm 144	16 \pm 1	ND	29 \pm 2	5.1 \pm 0.3
DBSPM 24h	1.04 \pm 0.05	98 \pm 9	1359 \pm 119	2419 \pm 149	962 \pm 39	NA	50 \pm 3	NA	14 \pm 1.6
DBSPM 48h	0.86 \pm 0.07	34 \pm 5	822 \pm 50	1824 \pm 52	1047 \pm 72	NA	36 \pm 2	NA	12 \pm 0.3

NA = not applicable, ND = not detectable