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ORIGINAL ARTICLE

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Induction of the PAOh1/SMO polyamine oxidase by polyamine analogues in human lung carcinoma cells

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Abstract *Purpose*: The induction of polyamine catabolism has been directly associated with the cytotoxic response of various tumor types to the antitumor polyamine analogues. Initially, human polyamine catabolism was assumed to be under the control of a rate-limiting spermidine/spermine N^1-acetyltransferase (SSAT) that provides substrate for an acetylpolyamine oxidase (PAO). We have recently cloned a new polyamine analogue-inducible human polyamine oxidase (PAOh1/SMO) that efficiently uses spermine as a substrate. The induction of PAOh1/SMO in response to multiple polyamine analogues was examined in representative lung tumor cell lines. *Methods*: Representatives of three different classes of antitumor polyamine analogues were examined for their ability to induce PAOh1/SMO. *Results*: The human adenocarcinoma line, NCI A549 was found to be the most responsive line with respect to induction of PAOh1/SMO in response to analogue exposure. Similar to previous observations with SSAT expression, PAOh1/SMO induction was found to occur primarily in non-small-cell lung cancers cell lines. Using a series of polyamine analogues, it was found that the most potent inducers of PAOh1/SMO possessed multiple three-carbon linkers between nitrogens, as typified by N^1,N^{11}-bis(ethyl)norspermine. *Conclusions*: Since PAOh1/SMO is an analogue-inducible enzyme that produces H_2O_2 as a metabolic product, it may play a significant role in determining the sensitivity of various human tumors to specific polyamine analogues.

Keywords Oxidation · Reactive oxygen species · Polyamine analogues · PAO · Spermidine/spermine N^1-acetyltransferase

Abbreviations *BENSpm* N^1,N^{11}-bis(ethyl)norspermine · *CHENSpm* N^1-ethyl-N^{11}-(cycloheptyl)methyl-4,8, diazaundecane · *CPENSpm* N^1-ethyl-N^{11}-(cyclopropyl)methyl-4,8,diazaundecane · *DAO* Diamine oxidase · *IPENSpm* (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8, diazaundecane · *MAO* Monoamine oxidase · *MDL 72,527* (N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine) · *PAO* Acetylpolyamine oxidase · *PAOh1/SMO* Human polyamine oxidase h1/spermine oxidase · *SSAT* Spermidine/spermine N^1-acetyltransferase

Introduction

Based on the ubiquitous requirement of tumor cell growth for polyamines, polyamine metabolism has been intensely investigated as a target for antineoplastic therapy [7, 26]. Although much work has focused on inhibiting the biosynthesis of polyamines through the rate-limiting biosynthetic enzymes ornithine decarboxylase and S-adenosylmethionine [30], the development of several antitumor polyamine analogues has resulted in increased interest in the regulation of polyamine catabolism [1, 2, 6, 7, 8, 17, 19, 33]. The interest in polyamine catabolism increased with the discovery that spermidine/spermine N^1-acetyltransferase (SSAT), a rate-limiting step in polyamine catabolism, is associated with the cell type-specific cytotoxic activity of some polyamine

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analogues [8, 10, 27, 31]. The increase in SSAT activity produces a rapid increase in acetylated polyamines which then become substrates of an acetylpolyamine oxidase (PAO) [36, 41]. It was originally hypothesized that H_2O_2 production from this last step in the two-step polyamine catabolism was responsible for cytotoxicity in some cases [12, 20]. However, our recent cloning of a new polyamine oxidase (PAOh1/SMO) that can efficiently use spermine as a substrate, and is inducible by polyamine analogues, has revealed a new and potentially exploitable target in polyamine metabolism [42]. Similar to the activity of PAO, PAOh1/SMO results in the production of H_2O_2, a reactive oxygen species that has been directly linked with the phenotype-specific toxicity of specific antitumor polyamine analogues [36].

In this study the induction of PAOh1/SMO by multiple antitumor polyamine analogues was examined in multiple lung cancer cell types representative of the various phenotypes of human lung cancer to demonstrate that PAOh1/SMO induction, similar to the induction of SSAT, is a phenotype-specific response to the analogues [8, 9, 10, 31, 32, 37]. The results also suggest that, similar to the effects observed with the high induction of SSAT, the high induction of PAOh1/SMO may also be linked with analogue-induced cytotoxicity for specific analogues.

Materials and methods

Chemicals and reagents

N^1,N^{11}-bis(ethyl)norspermine (BENSpm) was provided by Parke-Davis (Ann Arbor, Mich.) and N^1-ethyl-N^{11}-(cyclopropyl) methyl-4,8,diazaundecane (CPENSpm), N^1-ethyl-N^{11}-(cycloheptyl)methyl-4,8,diazaundecane (CHENSpm), (S)-N^1-(2-methyl-1-butyl)-N^{11}-ethyl-4,8,diazaundecane (IPENSpm), SL-11150,

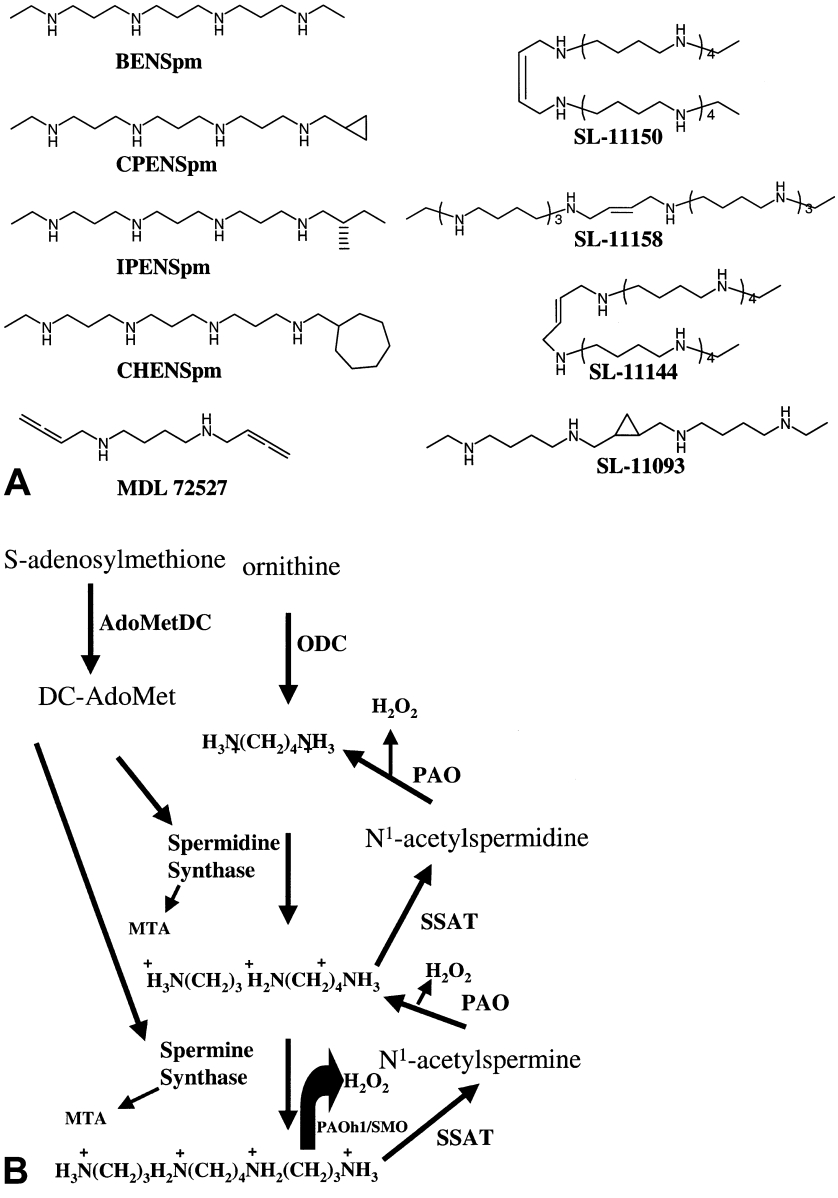


Fig. 1 **A** Antitumor polyamine analogues and PAOh1 inhibitor used in this study. **B** The polyamine metabolic pathway (*AdoMetDC* S-adenosylmethionine decarboxylase, *ODC* ornithine decarboxylase, *MTA* methylthioadenosine, *SSAT* spermidine/spermine N^1-acetyltransferase, *PAOh1/SMO* polyamine oxidase h1/spermine oxidase, *PAO* N^1-acetylpolyamine oxidase)

SL-11158, SL-11144, and SL-11093, and the selective PAOh1/SMO inhibitor MDL 72,527 were synthesized as previously reported [3, 4, 34, 35, 39, 44] (Fig. 1). N^1-Acetylspermine was purchased from Fluka (Buchs, Switzerland). Stock solutions (10 m*M*) of the various analogues were prepared in ddH_2O and stored at −20°C. Other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.), Invitrogen/Life Technologies (Rockville, Md.), Bio-Rad (Hercules, Calif.), Aldrich Chemical Company (Milwaukee, Wis.), Hyclone (Logan, Utah), and J.T. Baker (Phillipsburg, N.J.).

Cell culture and analysis of growth inhibition of human lung cancer cell lines

The non-small-cell cancer lines NCI A549 (adenocarcinoma), NCI H157 (squamous), NCI H727 (carcinoid), NCI H125 (adenocarcinoma) and U1752 (squamous), and the small-cell lung carcinoma lines NCI H82 and NCI H889 were cultured as we have previously described [10, 25]. Cells were treated for the times and with the concentrations of specific agents as indicated in the Results. For cell growth analysis in the presence of the PAOh1/SMO inhibitor MDL 72,527, the MTS dye reduction assay CellTiter 96 system from Promega was used according to the supplier's protocol. In these experiments, NCI A549 cells were seeded at 5×10^3 cells/well in a 96-well microtiter plate and treated for 96 h with 10 μ*M* of the indicated analogue in the presence or absence of 250 μ*M* MDL 72,527.

RNA purification and Northern blot analysis

Total cellular RNA was extracted from the lung cancer cell lines using Trizol reagent (Invitrogen) according to the manufacturer's protocol. For Northern blotting, total RNA (20 μg) was separated on a denaturing 1.5% agarose gel containing 6% formaldehyde and transferred to Zetaprobe membrane (Bio-Rad). Random primer-labeled PAOh1 cDNA was used as probe to estimate PAOh1/SMO expression [42]. Blots were stripped and reprobed with an 18S ribosomal cDNA to provide a loading control.

Analysis of polyamine content, SSAT and PAOh1/SMO activity

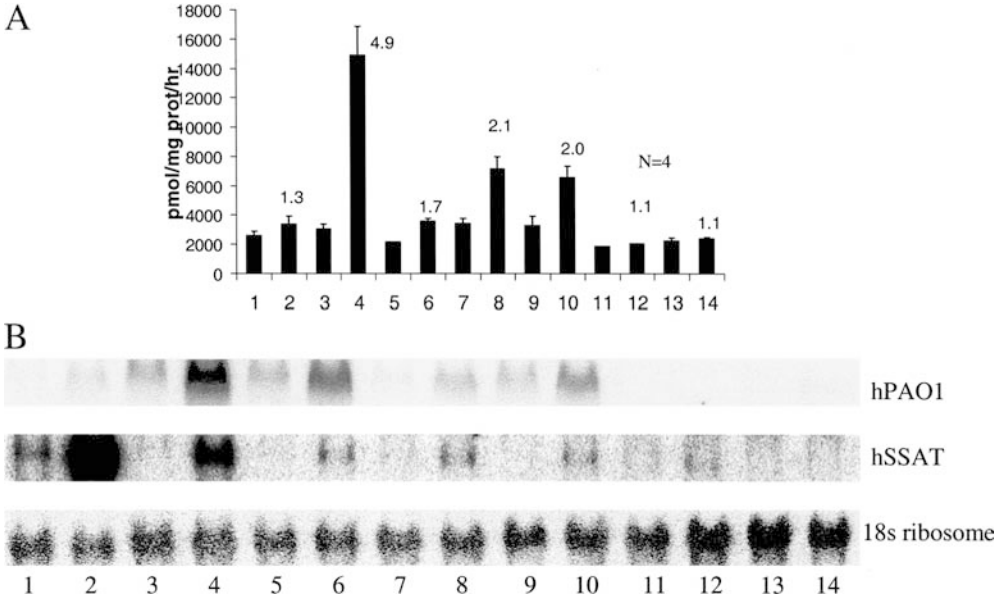
Intracellular polyamine concentrations were determined using the precolumn dansylation labeling, reverse-phase high-pressure liquid chromatography method as described by Kabra et al. [22] using 1,7-diaminoheptane as an internal standard. Polyamine concentrations are reported as nanomoles per milligram protein for each sample, where lysate protein content was measured by the method of Bradford [5]. SSAT activity of cellular extracts was measured as previously described [8]. The PAOh1/SMO enzyme activity in the cell lysates was assayed as previously described [42] by the method of Suzuki et al. [38] using 250 μ*M* spermine as the substrate. The PAOh1/SMO assays were performed in the presence of 1.0 m*M* pargyline and 0.1 m*M* semicarbizide as inhibitors of monoamine oxidase (MAO) and diamine oxidase (DAO), respectively.

Fig. 2 **A** Induction of PAOh1/SMO activity by BENSpm in human lung cancer cells. Seven human lung cancer cell lines representative of the major forms of lung cancer were exposed to 10 μ*M* BENSpm for 24 h to determine the effect on PAOh1/SMO activity. The numbers above each cell line represent the fold-increase in activity induced by BENSpm over untreated basal activity. The cell lines are: *1* H157 untreated, *2* H157 + BENSpm, *3* A549 untreated, *4* A549 + BENSpm, *5* H727 untreated, *6* H727 + BENSpm, *7* H125 untreated, *8* H125 + BENSpm, *9* U1752 untreated, *10* U1752 + BENSpm, *11* H889 untreated, *12* H889 + BENSpm, *13* H82 untreated, *14* H82 + BENSpm. Values are the means ± SE of two trials performed in duplicate using 250 μ*M* spermine as the substrate. **B** Representative Northern blot illustrating the effects of 24 h exposure to 10 μ*M* BENSpm on the expression of steady-state PAOh1/SMO mRNA. Each lane contained 20 μg total RNA isolated from each cell line. The lanes correspond to the designations in **A**. Note that the same blots were stripped and reprobed with an SSAT cDNA probe for comparison of relative mRNA levels

Results

PAOh1/SMO expression in human lung cancer cell lines in response to BENSpm exposure

BENSpm was chosen for the majority of studies reported here because it is one of the polyamine analogues that has been examined clinically [21] and because our initial studies indicated that PAOh1/SMO mRNA and PAOh1/SMO activity increases in a non-small-cell lung cancer line after 24 h exposure to 10 μ*M* BENSpm [42]. Therefore we examined the ability of BENSpm to induce PAOh1/SMO in seven human lung cancer cell lines that represent the major phenotypes of lung cancer (Fig. 2).



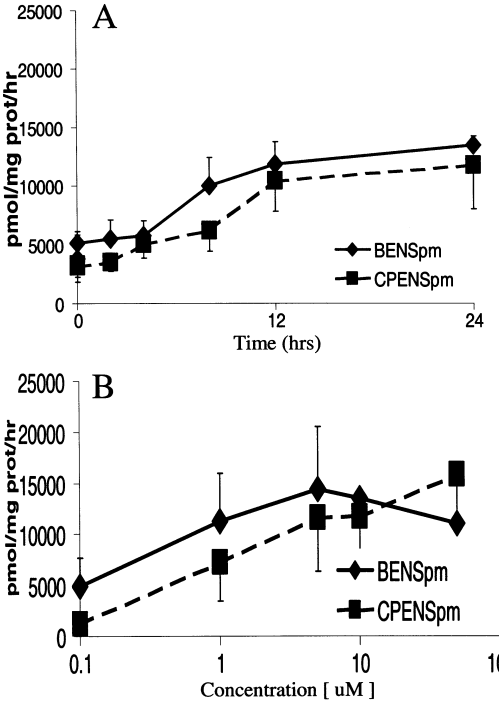


Fig. 3A, B Time- and dose-dependent changes in PAOh1/SMO activity in NCI A549 non-small-cell lung cancer cells in response to treatment with BENSpm or CPENSpm. **A** Cells were exposed to 10 μM BENSpm or CPENSpm for up to 24 h. **B** Cells were exposed to increasing concentrations of either BENSpm or CPENSpm for 24 h. The results in both **A** and **B** are the means ± SE of four separate experiments using 250 μM spermine as substrate performed in duplicate

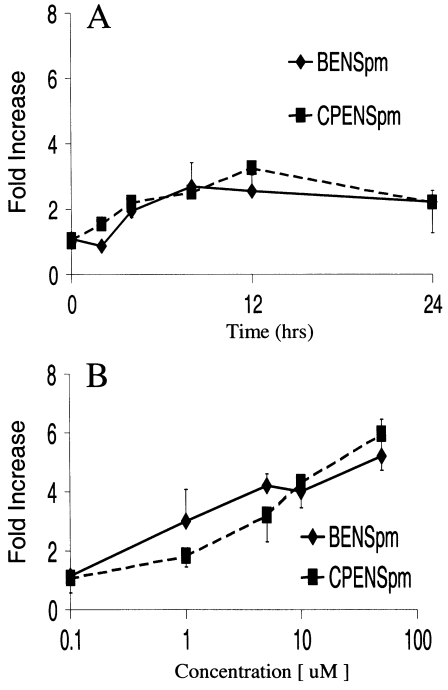


Fig. 4A, B Time- and dose-dependent changes in steady-state PAOh1/SMO mRNA levels in NCI A549 non-small-cell lung cancer cells in response to treatment with BENSpm or CPENSpm. **A** Increase in PAOh1/SMO mRNA with increasing exposure time to 10 μM analogue. **B** Increase in PAOh1/SMO mRNA with increasing concentrations of analogue for 24 h. Fold increase is relative to untreated controls. Each point represents the mean ± SE of two experiments performed in duplicate

Five cell lines exhibited modest to significant induction of PAOh1/SMO activity (Fig. 2A), with the adenocarcinoma line NCI A549 exhibiting the highest fold-induction of PAOh1/SMO activity (about fivefold). It should be noted that the basal levels of oxidase activity do not directly reflect the amount of specific PAOh1/SMO activity since less than 10% of the basal level is inhibited by MDL 72,527 (see below). Consequently, the fold-induction estimates most probably underestimate the actual fold-induction. The observed increases in PAOh1/SMO activity in the individual cell types were reflected by similar increases in steady-state PAOh1/SMO mRNA levels (Fig. 2B). There was no observed increase in PAOh1/SMO activity or mRNA in the two small-cell lung cancer lines examined.

Time- and dose-dependency of analogue-induced PAOh1/SMO activity

Since the NCI A549 cell line demonstrated the largest induction of PAOh1/SMO activity in response to BENSpm treatment, this line was chosen for further testing. CPENSpm treatment was also performed since it has also been shown to induce SSAT in a manner similar to BENSpm in these cells [11]. The effects of increasing time of BENSpm exposure on PAOh1/SMO activity were readily observed (Fig. 3A) when cells were exposed to 10 μM BENSpm for 0.5 to 24 h. The activity had increased approximately threefold by 12 h and nearly fivefold by 24 h. When NCI A549 cells were exposed to increasing concentrations of BENSpm for 24 h, the maximal induction of PAOh1/SMO was observed at 5 μM with lower activity at higher concentrations (Fig. 3B). Nearly identical results were observed when cells were exposed to the polyamine analogue CPENSpm, with the exception that PAOh1/SMO activity continued to increase at concentrations up to 50 μM CPENSpm (Fig. 3). The increase in PAOh1/SMO activity was generally accompanied by an increase in the 2.4 kb steady-state PAOh1/SMO mRNA (Fig. 4). It should be noted that the NCI A549 cells readily accumulated both BENSpm and CPENSpm, resulting in a decrease in intracellular polyamine pools and an induction of SSAT activity (Table 1). We have previously demonstrated that both compounds are cytotoxic to non-small-cell lung cancer lines after 96 h exposure to concentrations >1 μM [7, 11]. To determine whether the inhibition of PAOh1/SMO activity could alter the response of NCI A549 cells to BENSpm or CPENSpm, cell growth studies were performed where the cells were simultaneously exposed to the analogue and the PAOh1/SMO inhibitor MDL 72,527 and cultured for 96 h (Fig. 5). There was a clear decrease in sensitivity to

Table 1 Effects of 24-h analogue treatment of NCI A549 cells on polyamine pools, PAOh1/SMO and SSAT activities. Values are means ± SE, four experiments in duplicate for the polyamines, and four experiments in triplicate for PAOh1/SMO and SSAT (*SSAT* spermidine/spermine N^1-acetyltransferase)

Treatment	Polyamine (nmol/mg protein)			Analogue (nmol/mg protein)	PAOh1/SMO (pmol H_2O_2/mg/h)	SSAT (pmol N^1-acetylspermidine/mg protein/min)
	Putrescine	Spermidine	Spermine			
None	2.1 ± 0.7	26.9 ± 1.2	9.9 ± 1.2	N/A	6,390 ± 2,070	5 ± 0.1
10 μ*M* BENSpm	< 0.05	1.2 ± 0.8	1.2 ± 0.3	49.4 ± 9.8	16,100 ± 780	5590 ± 517.7
10 μ*M* CPENSpm	< 0.05	5.5 ± 0.2	2.4 ± 0.4	17.8 ± 1.4	14,300 ± 3,700	1190 ± 287.9

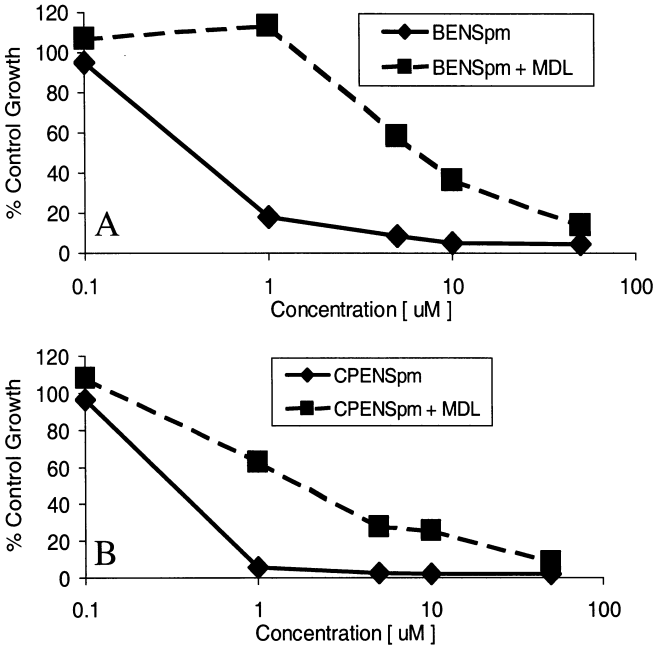


Fig. 5A, B Effects of cotreatment of NCI A549 cells with PAOh1/SMO-inducing analogues and the PAOh1/SMO inhibitor MDL 72,527. Cells were seeded at 5×10^3 cells/well and treated for 96 h with (**A**) 10 μ*M* BENSpm or (**B**) 10 μ*M* CPENSpm in the presence or absence of 250 μ*M* MDL 72,527 (*MDL*). Each point represents the mean ± SD of triplicate determinations. Note that the error bars fall within the symbol at each point

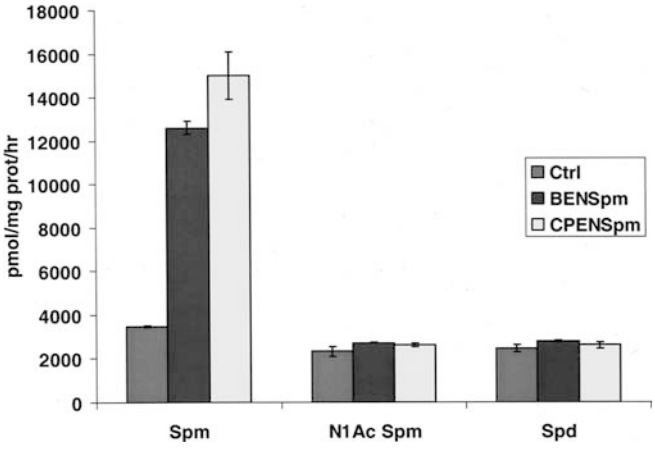


Fig. 6 Substrate specificity of PAOh1/SMO activity induced by BENSpm and CPENSpm. NCI A549 cells were treated with 10 μM of either BENSpm or CPENSpm for 24 h. Cell lysate from the treated cells were then assayed for PAOh1/SMO activity using 250 μ*M* spermine, spermidine, or N^1-acetylspermine. The data represent the means of four separate experiments performed in duplicate ± SE

both analogues in the presence of the PAOh1/SMO inhibitor.

Substrate specificity of analogue-induced PAOh1/SMO in NCI A549 cells

Vujcic et al. have recently reported that in a transfection system using an expression vector containing virtually the identical sequence we originally identified as PAOh1, the resulting lysate could only efficiently use spermine as a substrate [40]. To determine the substrate specificity of the PAOh1/SMO activity induced in the NCI A549 cells, cell lysates from cells treated with either 10 μ*M* BENSpm or 10 μ*M* CPENSpm for 24 h were examined for their ability to catalyze spermine, spermidine, and N^1-acetylspermine (Fig. 6). This concentration was chosen since it has previously been demonstrated to significantly induce polyamine catabolism through SSAT and because it is an attainable concentration of BENSpm in the clinical setting [9, 21]. The results clearly indicated that only spermine was a substrate for the inducible PAOh1/SMO activity in the NCI A549 lysates.

Induction of PAOh1/SMO by polyamine analogues is structure dependent

To determine the basic structural requirements of PAOh1/SMO induction in NCI A549 cells, the ability of eight polyamine analogues that are undergoing or are being considered for clinical trials were examined for their ability to induce PAOh1/SMO activity. The symmetrically substituted BENSpm, and the asymmetrically substituted CPENSpm, CHENSpm, and IPENSpm led to significant induction of PAOh1/SMO after 24 h exposure to 10 μ*M* of each analogue (Fig. 7). The oligoamine analogues, SLIL 11144, 11150, 11158, and the conformationally restricted analogue, SLIL 11093, did not induce PAOh1/SMO. The results of these studies suggest that one structural requirement for PAOh1/SMO induction is the presence of multiple aminopropyl moieties within the analogue structure. It is also

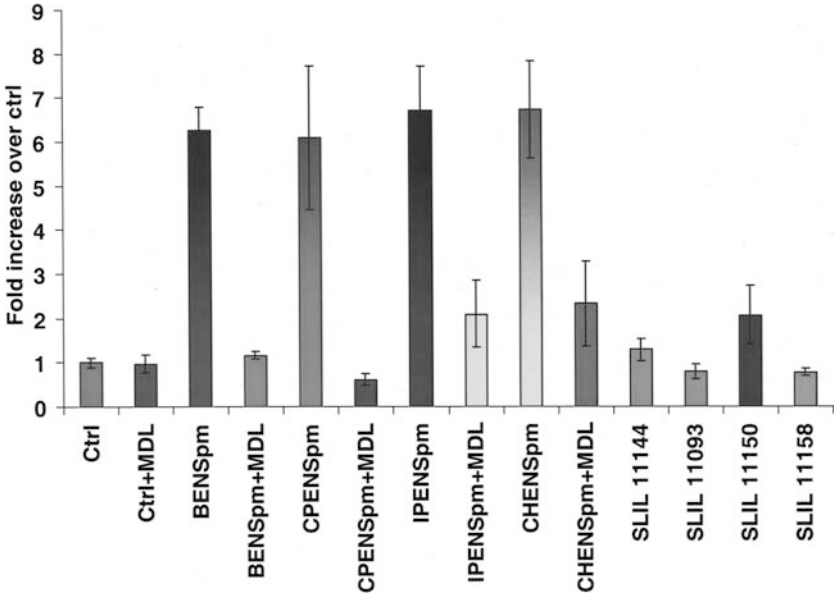


Fig. 7 Ability of various polyamine analogues to induce PAOh1/SMO activity. NCI A549 cells were exposed to 10 μ*M* of the indicated polyamine analogues for 24 h. PAOh1/SMO activity was then measured in the corresponding cell lysates using 250 μ*M* spermine as the substrate. The basal oxidase activity was 4184 pmol/mg per hour. It should be noted that 250 μ*M* MDL 72,527 (*MDL*) only reduced the basal activity to 4055 pmol/mg per hour. The PAOh1/SMO inhibitor MDL 72,527 was used at a concentration of 250 μ*M* where indicated (*MDL*) in the assay of the analogue-induced PAOh1/SMO to determine if this induced activity could be efficiently inhibited. The results presented are the means ± SE of four separate experiments performed in duplicate

important to note that the polyamine oxidase inhibitor MDL 72,527 significantly inhibited the PAOh1/SMO activity induced by the analogues, but did not significantly reduce the basal levels of oxidase activity. These data are consistent with the possibility that there is a basal oxidase activity in the NCI A549 cells that is not inducible by polyamine analogues and is not inhibited by PAOh1/SMO, MAO, or DAO inhibitors.

Discussion

The role of polyamine catabolism in response to antitumor polyamine analogues and other agents has become an intense area of investigation based on the discovery that it is, in some instances, causally associated with the cell type-specific cytotoxicity of these agents [12, 20, 24, 29]. However, the study of polyamine catabolism in mammalian cells had previously been limited since no mammalian polyamine oxidases had been cloned. Our laboratory helped fill this deficiency by providing the first clone of a human polyamine oxidase (PAOh1) that could readily use spermine as a substrate [42]. Here data are presented which demonstrate that this newly characterized enzyme is inducible in a tumor cell type-specific and agent-specific manner. These results suggest that PAOh1/SMO activity can have an effect on tumor cell response to specific antitumor polyamine analogues.

Similar to results observed with analogue-induction of SSAT, the non-small-cell lung cancer phenotypes responded to analogue exposure with a higher induction of PAOh1/SMO than did the small-cell lung cancer phenotypes. However, it should be noted that unlike SSAT, which is most highly expressed in the non-small-cell lung cancer line NCI H157, PAOh1/SMO was found to be most highly expressed in the adenocarcinoma cell line NCI A549, at the levels of both mRNA (Fig. 2) and activity [10]. The results indicating that PAOh1/SMO is an inducible enzyme are significant since previous data suggest that the oxidation of polyamines is limited by the availability of acetylated substrate [36]. However, in the NCI A549 cells the inducible PAOh1/SMO activity clearly preferred spermine as the substrate, and was not significantly active on either N^1-acetylspermine or spermidine (Fig. 6). Additionally, preliminary studies demonstrated that induction of PAOh1/SMO activity by the polyamine analogues occurs in a phenotype-specific manner in a number of human tumor cell types including breast, prostate and colon cancer cell lines [16].

These results are consistent with those recently reported by Vujcic et al. [40] who used a transfection model to demonstrate a spermine preference by an essentially identical clone (identified as SMO by Vujcic et al.) to the PAOh1h1 clone we originally reported. It is important to note that during the preparation of this report, Vujcic et al. [41] reported the identification of a mammalian oxidase that preferentially oxidizes acetylated polyamines. It should also be stressed that multiple splice variants of PAOh1 have been identified and each appears to possess different kinetic properties [28]. However, more study is necessary to determine the spectrum of expression of these splice variants in normal and tumor cells in order to determine the extent of their physiological relevance.

Each of the key enzymes in polyamine metabolism has been demonstrated to be regulated at multiple levels [26, 30]. SSAT is known to be significantly post-transcriptionally regulated. The levels of SSAT protein induced by polyamine analogues are often in excess of those expected by observed increases in SSAT mRNA [13, 14, 15, 18]. Interestingly, in the case of PAOh1/SMO, the increase in steady-state PAOh1/SMO mRNA closely parallels the observed increase in PAOh1/SMO activity. This parallel increase in message and activity was seen in both the time- and dose-dependent studies. These results suggest that PAOh1/SMO may be primarily regulated by changes in mRNA levels; however, formal transcriptional studies will have to be performed to determine if transcription is the key regulatory step.

The induction of PAOh1/SMO in A549 cells appears to be agent-specific. Interestingly, the agents that were demonstrated to be the best inducers of SSAT (BENSpm, CPENSpm, etc.) also, with one exception, appear to be the best inducers of PAOh1/SMO [7]. The one exception is CHENSpm, which did not significantly induce SSAT in most cell types, but clearly was an effective inducer of PAOh1/SMO in the A549 cells. It should be noted that CHENSpm has been shown to be a substrate of a polyamine oxidase activity in CHO cells [23]. However, it is likely that this oxidase activity is the classical acetylpolyamine oxidase PAO recently cloned by Vujcic et al. [40] and not PAOh1/SMO activity. Importantly, Wang et al. [43] have demonstrated that none of the analogues used in this study is an effective substrate for purified PAOh1/SMO.

The small number of conformationally restricted and oligoamine analogues used in this study did not demonstrate an ability to induce PAOh1/SMO. Based on these data, it appears that the presence of three-carbon bridges between nitrogens as exist in BENSpm and CPENSpm are critical to PAOh1/SMO induction. These results, although derived from only a small number of analogues, are significant since there is increasing interest in the development of antitumor polyamine analogues and because the oxidation of polyamines can play a significant role in determining the relative sensitivity of particular tumor types to the analogues. This role is particularly evident in the studies demonstrating that the inhibition of PAOh1/SMO activity by MDL 72,527 significantly alters the dose response of NCI A549 in response to both BENSpm and CPENSpm requiring greater concentration of analogue to produce the growth effects. However, it is important to note that the concentration of MDL 72,527 used here did not adversely affect the growth of NCI A549 cells. It should also be emphasized that these oligoamines and conformationally restricted analogues have demonstrated significant in vitro activity against human prostate and breast cancer cells [34, 35] and in our lung cancer cell panel (unpublished observations). Therefore, PAOh1/SMO induction may be one component of specific analogue toxicity, but it is clearly not a requirement for all analogue activity.

Polyamine metabolism continues to be a focus of promising antineoplastic drug development. The recent discovery of a new enzyme in the polyamine catabolic pathway that produces H_2O_2, a mediator of toxicity that has been directly linked to tumor cell response, provides yet another potentially exploitable target. Although further study is required to determine the full potential of drug-induced modulation of PAOh1/SMO, the current results present an important start in understanding the role of PAOh1/SMO in defining tumor sensitivity to specific agents.

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