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Growth and biochemical effects of unsymmetrically substituted polyamine analogues in human lung tumor cells1

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Abstract Three unsymmetrically substituted polyamine analogues demonstrate significant and selective antitumor effects. Each of the analogues N^1-ethyl-N^{11}-propargyl-4,8-diazaundecane (PENSpm); N^1-ethyl-N^{11}-(cyclobutyl)methyl-4,8-diazaundecane (CBENSpm), and N^1-ethyl-N^{11}-(cyclopropyl)methyl-4,8-diazaundecane (CPENSpm) is cytotoxic to a representative non-small-cell lung carcinoma line, NCI H157, while being only growth-inhibitory to a representative small-cell-lung carcinoma line, NCI H82. Cytotoxicity is accompanied by a significant increase in expression of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) at the levels of activity and steady-state mRNA. These new analogues are significant both for their cell-type-specific activity and as synthetic prototypes for the addition of SSAT-activated functional groups.

Key words PENSpm · CBENSpm · CPENSpm · SSAT activity · mRNA expression

Introduction

The naturally occurring cationic polyamines are known to be required for cell growth and differentiation [18]. In the last several years this pathway has been studied as a potential target of antineoplastic intervention [19, 22]. Most work has focused on the synthesis and testing of specific inhibitors of the biosynthetic pathway. Although this work has led to an increased understanding of the requirements and mechanisms of action of the polyamines, it has resulted in little clinical success [26]. Recently, Porter et al. embarked on the synthesis and study of structural analogues of the natural polyamines as agents that were designed to mimic the natural polyamines in their self-regulatory mechanisms while not substituting functionally for cellular requirements [7, 23, 28].

We originally described an unusual cytotoxicity to one class of these agents, the bis(ethyl)polyamines [8, 9]. A cell-type-specific activity of these compounds across a spectrum of human lung tumor and melanoma cell lines has also been described [8, 12, 24]. In each case there appears to be an association between the ability of the individual cell type to superinduce the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) and the selectivity of the bis(ethyl)polyamines [8, 12, 24, 30]. SSAT is the rate-limiting enzyme of the polyamine catabolic pathway and is inducible in response to several agents and treatments [6, 29]. However, only in relatively few cell types is this enzyme superinduced to levels that can exceed 10^3 times the basal level in response to certain agents [12, 24].

On the basis of these data we proceeded to synthesize and evaluate members of a new class of unsymmetrically substituted polyamine analogues for their potential antitumor activity. The analogues, N^1-ethyl-N^{11}-propargyl-4,8-diazaundecane (PENSpm), N^1-ethyl-N^{11}-(cyclobutyl)-methyl-4,8-diazaundecane (CBENSpm) and N^1-ethyl-N^{11}-(cyclopropyl)methyl-4,8-diazaundecane (CPENSpm; Fig. 1) [25] were examined for their growth-inhibitory effects and their effects on polyamine metabolism. As an initial test system we chose two representative human lung-cancer cell lines that have been useful in discovering the differential activity of compounds that interfere with polyamine metabolism [9]. The results of the current studies suggest the possibility of changing the basic structure of the symmetrically substituted bis(ethyl)polyamines while maintaining or improving upon their cell-type specificity. Furthermore, the synthetic scheme leading to the current compounds [25] may also allow an improvement over the symmetric compounds by allowing the addition of monofunctional groups. It is projected that this strategy will aid our

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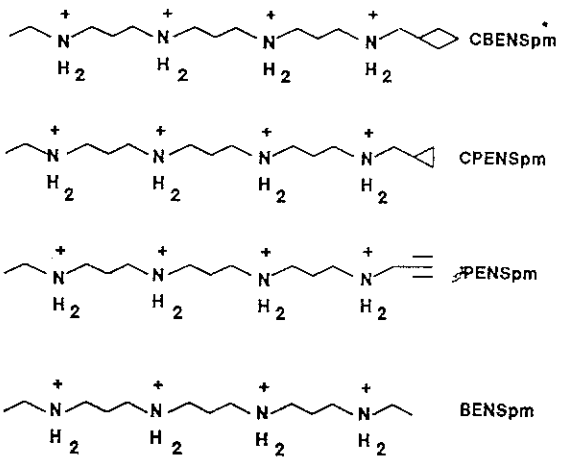


Fig. 1 The structure of the unsymmetrically substituted polyamine analogues as compared with the symmetrically substituted BENSpm

understanding of the mechanisms of action underlying the observed differential regulation of the SSAT gene and will potentially provide insight into the role of induced SSAT in the cytotoxic response of human tumor cells to polyamine analogues. Preliminary biological effects of PENSpm and CPENSpm have been reported elsewhere [25].

Materials and methods

Chemicals

PENSpm, CBENSpm, and CPENSpm were synthesized as previously reported [25]. BENSpm was prepared according to Bergeron et al. [1–3] and were kindly provided by the SunPharm Corp. (Jacksonville, Fla.). The compounds were prepared as 10-m*M* stock solutions in 0.1 *M* HCl and were diluted in media for cell treatment as described below.

Cell culture

The representative cell lines, large-cell undifferentiated lung carcinoma, NCI H157 and small-cell lung carcinoma (SCLC) NCI H82, were maintained in culture as previously reported [9]. For determination of drug effects, the cultures were manipulated as described in Results. After treatment, cells were harvested and assayed for cell growth, polyamine content, SSAT activity, and mRNA levels as described below. Cell viability was determined by trypan blue exclusion. The results of cell-growth studies are reported as a ratio of the number of viable cells (N) detected at the indicated times divided by the number of cells originally seeded (N_0). Both cell types were seeded at the density of 2×10^6 cells/75-cm^2 flask. Log-phase growth was maintained in control NCI H157 and NCI H82 cells during the 96-h culture period. The definition of cytotoxicity used in the current work is consistent with that previously published by ourselves and other investigators and is said to correspond to $N/N_0 < 1$ [9, 16, 17]. It should be noted that the definition of cytotoxicity used herein is equivalent to net cell loss from culture and suggests that cell death is occurring at a greater rate than cell division. It does not imply the absence of cell division.

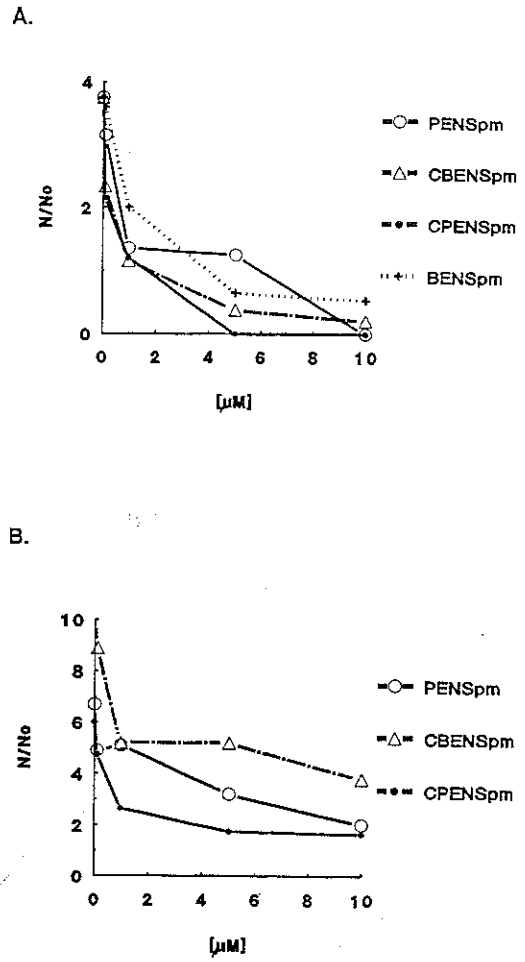


Fig. 2A,B Dose response of representative human lung tumor cells to the unsymmetrically substituted polyamine analogues PENSpm, CBENSpm, and CPENSpm. A NCI H157 non-SCLC and B NCI H82 SCLC cells were exposed to increasing concentrations of the compound incicated. The effects of BENSpm on NCI H157 are included for comparison. Each point represents the mean value for duplicate experiments

Analysis of polyamine content and of SSAT and ornithine decarboxylase activities

The polyamine content of treated and untreated cells was determined by precolumn dansylation/reversed-phase high-performance liquid chromatography [15] using 1,7-diaminoheptane as the internal standard. This method is sufficiently sensitive to detect >5 pmol of the individual polyamines and is generally reproducible within 15% of variation. SSAT and ornithine decarboxylase (ODC) activities were measured using cellular extracts as previously reported [10, 27]. Protein concentrations were determined by the method of Bradford [5].

Northern analysis

Total cellular RNA from treated and untreated cells was extracted and used for Northern analyses as previously published [11, 14]. The probes used for hybridization were the full-length human SSAT cDNA clone AP3/F7 [32], the human ODC cDNA clone pODC 10/2 h [31], and a homologous rat glyceraldehyde-3-phosphate dehydrogenase cDNA [21].

Results

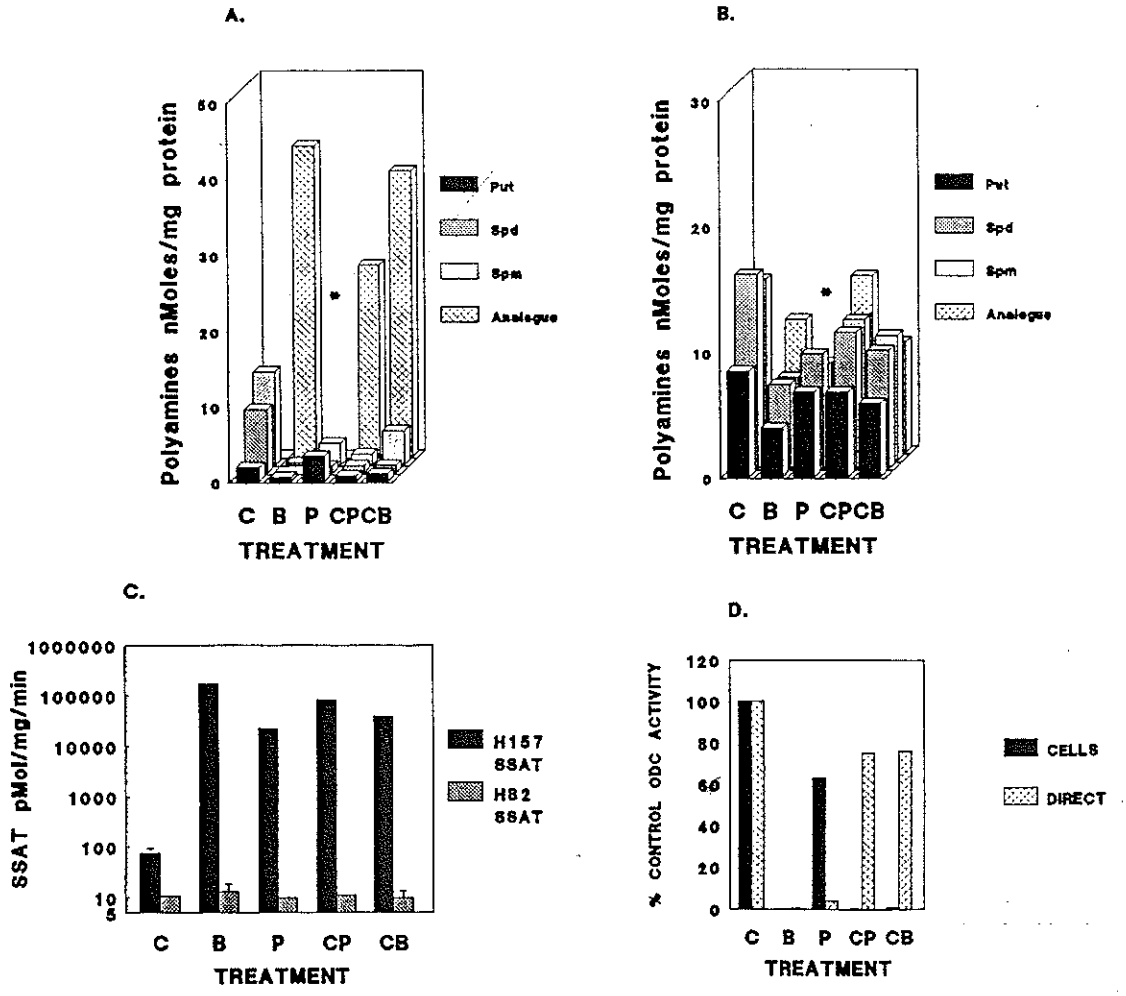
Differential cytotoxicity

The human SCLC line NCI H82 and the human large-cell lung carcinoma (non-SCLC) line NCI H157 were chosen for testing because these two cell lines have proved to be useful predictors of disparate sensitivity to agents that interfere with the polyamine metabolic pathway. As stated above, these two lines respond very differently to treatment with the symmetrically substituted bis(ethyl)polyamine compounds. To compare the two cell types for their sensitivity to the new unsymmetrically substituted analogues, we performed 96-h dose-response experiments within a concentration range of 0–10 μ*M* for each compound (Fig. 2). The dose response of NCI H157 cells to BENSpm is included for comparison. The non-SCLC H157 line was found to be significantly more sensitive to the polyamine depletion effects (Figs. 2a, 3a) and cytotoxic ($N/N_o < 1$) effects of the compounds. Minimal polyamine depletion and no cytotoxicity was observed in the H82 SCLC line within the concentration range tested. However, growth inhibition was observed after 96 h treatment with each analogue. For both cell types, CPENSpm appears to be the most growth-inhibitory of the compounds within the concentration range tested.

Induction of SSAT activity

Since cell-type-specific sensitivity appears to correlate with the differential ability of the cells to exhibit SSAT super-

Fig. 3A–D Effects in NCI H157 and NCI H82 cells after 24 h treatment with BENSpm (*B*), PENSpm (*P*), CPENSpm (*CP*), and CBENSpm (*CB*) on levels of polyamines and on SSAT and ODC activity. NCI H157 and NCI H82 cells were treated for 24 h with 10 μ*M* of the compound indicated. **A** Effects of treatment on the polyamine concentration in H157 cells. **B** Effects of analogue treatment on the polyamine concentrations in H82 cells. Data represent the mean values for duplicate determinations. PENSpm (*) concentrations could not be reliably quantitated using standard methods. **C** Effects of polyamine analogue treatment on SSAT activity in H157 (*solid bars*) and H82 (*hatched bars*) cells. Data represent the mean values for triplicate determinations; error bars are indicated where standard deviations exceed 10%. **D** Effects of analogues on ODC activity in treated H157 cells (*solid bars*) and in direct-inhibition studies (*hatched bars*). Data represent the mean values for triplicate determinations. The control value for untreated cells (*solid bar*) was 13.35 nmol mg protein^{-1} h^{-1}. The control value for the untreated lysate used for direct-inhibition studies (*hatched bar*) was 8.5 nmol mg protein^{-1} h^{-1}



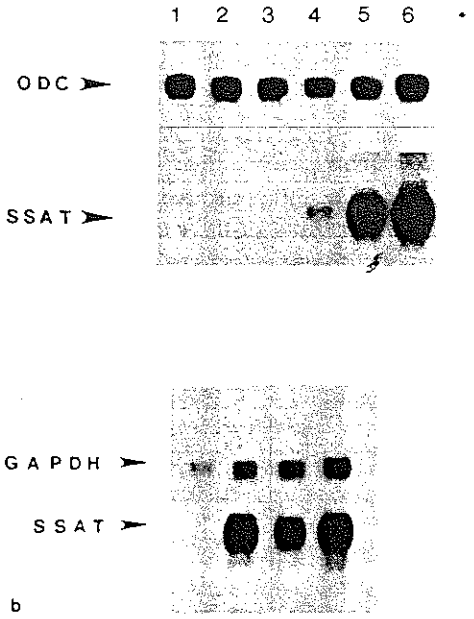


Fig. 4 a Effects of 24 h analogue treatment on ODC and SSAT steady-state mRNA levels in H157 and H82 cells. Blots were sequentially probed with probes homologous to human ODC and human SSAT (*Lane 1* H82 control, *lane 2* H82 10 μ*M* PENSpm, *lane 3* H82 10 μ*M* CPENSpm, *lane 4* control H157, *lane 5* 10 μ*M* PENSpm, *lane 6* 10 μ*M* CPENSpm). b A separate experiment comparing increases in SSAT mRNA in H157 cells treated for 24 h with the various analogues (*Lane 1* Control, *lane 2* 10 μ*M* BENSpm, *lane 3*, CBENSpm, *lane 4* 10 μ*M* CPENSpm). GAPDH was used as a determinant of relative RNA loading

induction, we examined the ability of each of the new compounds to induce SSAT at the level of enzyme activity and steady-state message (Fig. 3A, B). Similar to findings obtained with the bis(ethyl)polyamines, the H157 cells were found to respond to treatment with either compound with a significant induction of SSAT at the level of both mRNA and enzyme activity. Increases in steady-state SSAT mRNA levels paralleled the increase observed in enzyme activity and in NCI H157 cells (Fig. 4). By contrast, SSAT activity and mRNA levels of the SCLC H82 line were unaffected by the analogues (Figs. 3C, 4a). It is noteworthy that the NCI H82 line accumulates less analogue after 24 h treatment than NCI H157. Comparable amounts of the analogue are accumulated with longer treatment periods with no induction of SSAT (data not shown). These findings are consistent with results previously obtained in NCI H82 cells and the symmetric bis(ethyl)polyamines [12].

Effect on ODC activity

The ability of each of the analogues to down-regulate the polyamine biosynthetic enzyme ODC was examined. NCI H157 cells were treated for 24-h with 10 μ*M* of each compound (Fig. 3D). Each of the analogues produced significant decreases in cellular ODC activity. However, PENSpm treatment reduced activity by only 25%, whereas the other compounds reduced activity to nearly undetectable levels (Fig. 3D). Furthermore, to determine if the decrease in ODC activity was a result of direct inhibition of the enzyme, untreated H157 cell lysates containing high ODC activity were incubated with 3 m*M* of each compound. This concentration was chosen since the analogues can be accumulated to this extent in treated H157 cells. CPENSpm and CBENSpm produced inhibition of only ~25%. By contrast, the symmetric BENSpm inhibited activity to the level of detection. Interestingly, PENSpm, the least effective regulator of ODC in treated cells, also directly inhibited ODC activity substantially. CPENSpm and PENSpm had no effect on ODC mRNA in either the SCLC H82 line or the H157 line (Fig. 4a).

Discussion

The purpose of the current work was to determine if unsymmetrically substituted analogues of the polyamines would maintain the tumor-type specificity and activity of the symmetrically substituted bis(ethyl)polyamines. The polyamine metabolic pathway has become an increasingly attractive target for therapeutic intervention in the experimental treatment of cancer. Much of the renewed interest is a result of the unusual phenotype-specific cytotoxicity observed with the bis(ethyl)polyamines. These analogues have been shown to be effective against several important human solid tumor types both in vitro and in vivo [4, 12, 13, 24]. We have recently demonstrated an association between the superinduction of SSAT and the cytotoxic response of the non-SCLC human lung cancer phenotype [8]. The increase in SSAT activity occurs at the level of increased SSAT mRNA and new protein synthesis [10, 11]. Therefore, we attempted to exploit this apparent cell-specific response to polyamine analogues and began to examine compounds that may improve upon the bis(ethyl)polyamine paradigm and provide additional insight into the molecular mechanism of SSAT induction. Similar to the activity previously described for the symmetrically substituted polyamines [23], CPENSpm and CBENSpm are effective down-regulators of ODC activity while having little direct inhibitory effect. BENSpm and PENSpm are effective in directly reducing ODC activity when incubated with ODC-containing lysates, but cellular treatment with PENSpm produces only a limited reduction of ODC activity. Consistent with the results of Porter et al. [23], the decrease in ODC activity observed in treated cells does not appear to result from changes in steady-state ODC mRNA but may be due to translational effects as suggested by Pegg and colleagues [20].

CBENSpm, CPENSpm, and PENSpm were found to be highly active against the human large-cell lung carcinoma NCI H157. This activity was manifested by cell kill at concentrations as low as 5 μ*M* and significant growth inhibition was observed at concentrations as low as 1 μ*M*. Cytotoxic activity was not observed in the human SCLC line NCI H82. In this respect the new compounds behave

very similarly to BENSpm. This finding is significant in demonstrating that these compounds are not simply general toxins. However, significant growth inhibition was observed in NCI H82 cells treated with each compound, with CPENSpm demonstrating the greatest activity. It is noteworthy that although NCI H82 cells have a shorter generation time than NCI H157 cells, they are less affected by the analogues, suggesting that rapid cell growth is not a prerequisite for cytotoxic activity by these compounds.

With respect to the induction of SSAT, the current results demonstrate that both the symmetric and the unsymmetrically substituted compounds have the ability to superinduce SSAT at the level of protein and mRNA in the sensitive phenotype. Although there are differences between the analogues, abilities to induce SSAT activity and mRNA, the relative effectiveness of each compound in vitro is comparable with that of the others. These results further substantiate the hypothesis that there may be some threshold of SSAT activity above which cytotoxicity occurs [12, 30]. Potentially the greatest significance of the current work is the verification of sustained selective activity of the unsymmetrically substituted polyamine analogues. These compounds and the synthetic scheme used in their synthesis will provide the opportunity to design and synthesize compounds with functional groups on one terminus while maintaining the parent structure on the other. As one goal, we intend to synthesize compounds that both induce SSAT in a tumor-specific manner and are activated by the acetyltransferase to a more toxic compound, thereby specifically targeting only those cells capable of activating them.

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