Antizyme induction by polyamine analogues as a factor of cell growth inhibition

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The polyamines spermidine and spermine and their diamine precursor putrescine are essential for mammalian cell growth and viability, and strategies are sought for reducing polyamine levels in order to inhibit cancer growth. Several structural analogues of the polyamines have been found to decrease natural polyamine levels and inhibit cell growth, probably by stimulating normal feedback mechanisms. In the present study, a large selection of spermine analogues has been tested for their effectiveness in inducing the production of antizyme, a key protein in feedback inhibition of putrescine synthesis and cellular polyamine uptake. Bisethylhomospermine, bisethylhomospermine, 1,19-bis(ethylamino)-5,10,15-triazanonadecane, longer oligoamine constructs and many conformationally constrained analogues of these compounds were found to stimulate antizyme synthesis to

different levels in rat liver HTC cells, with some producing far more antizyme than the natural polyamine spermine. Uptake of the tested compounds was found to be dependent on, and limited by, the polyamine transport system, for which all these have approximately equal affinity. These analogues differed in their ability to inhibit HTC cell growth during 3 days of exposure, and this ability correlated with their antizyme-inducing potential. This is the first direct evidence that antizyme is induced by several polyamine analogues. Selection of analogues with this potential may be an effective strategy for maximizing polyamine deprivation and growth inhibition.

Key words: bisethylhomospermine, bisethylhomospermine, putrescine, spermidine, spermine.

INTRODUCTION

The polyamines spermidine and spermine and their diamine precursor putrescine are aliphatic cations, which are essential for the normal physiology and growth of almost all cells [1–3]. In mammalian tissues, polyamine homeostasis involves a composite of several sensitive feedback systems regulating the synthesis, degradation and transport of polyamines. Disruptions in this homeostasis, which lead to abnormal accumulation of the polyamines, contribute to tumour induction, growth and metastasis. Because of this role in cancer formation, there is currently intense interest in the design and testing of interventions to modulate cellular polyamine levels [1,2,4–10]. Specific inhibitors of polyamine biosynthesis have been produced, and at least one of them, α -difluoromethylornithine (eflornithine) (DFMO), is in multiple clinical trials, where its anticarcinogenic potential is being tested [11]. Similarly, the inhibition of cancer cell growth by reduction of tissue polyamine levels has also been achieved by a variety of polyamine structural analogues, but their mechanisms are not fully defined [1,2,10,12].

Many polyamine mimics have been synthesized with the expectation that some would efficiently gain entrance into cells via the polyamine transporter and then displace native polyamines in some critical cell function, or perhaps stimulate a feedback mechanism to down-regulate the native polyamines. The compound 1,19-bis(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4), e.g., has been shown to be highly cytotoxic and to be useful in decreasing the growth of certain brain tumours on account of its high affinity for DNA [13]. Another compound,

bisethylhomospermine (BENSPM), which has undergone phase II clinical trials for chemotherapeutic efficacy, is believed to deplete effectively cell polyamines by significantly stimulating the activity of the polyamine-degrading enzyme, spermidine/spermine acetyltransferase [14,15]. Many of the polyamine mimics decrease cell polyamines to different degrees by decreasing the activity of the initial enzyme in polyamine biosynthesis, ornithine decarboxylase (ODC) [16]. Where evaluated, decreases in polyamine uptake have also been noted [17]. Although such activities suggest that many analogues are inducing a normal polyamine feedback response, the mechanism has not been fully explored.

In normal mammalian cells, an increase in the levels of the polyamines stimulates the synthesis of a small regulatory protein called antizyme. As reviewed elsewhere [18–20], translation of antizyme mRNA requires an unusual, polyamine-dependent +1 frameshift. Once synthesized, antizyme binds ODC and facilitates its rapid degradation by 26 S proteasome in a ubiquitin-independent manner. Antizyme not only facilitates rapid inactivation of polyamine biosynthesis but mediates polyamine-induced feedback inhibition of the polyamine transport system [21,22]. As a central component of the feedback regulation of both pathways for increasing cellular polyamine concentrations, antizyme is crucial to normal polyamine homeostasis. It follows that abnormalities in antizyme production or activation may underlie the elevations in polyamine synthesis and uptake associated with many tumours. Previous reports have confirmed that antizyme levels are decreased in carcinogen-induced oral cancers [23], and faulty antizyme regulation has also been implicated in tumour progression in prostate carcinoma [24].

Abbreviations used: BE-4-4-4-4, 1,19-bis(ethylamino)-5,10,15-triazanonadecane; BEHSPM, bisethylhomospermine; BENSPM, bisethylhomospermine; DFMO, α -difluoromethylornithine (eflornithine); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ODC, ornithine decarboxylase.

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Consistent with these observations, enhanced antizyme production shows anti-tumour activity. Specifically, interleukin-1-induced growth inhibition of human melanoma cells has been found to be specifically associated with enhanced production of antizyme mRNA [25], and the increased antizyme production in H-ras-transformed NIH3T3 cells blocks their ability to form tumours in mice [26].

Since tumour formation depends on abnormal elevations in cellular polyamines, which are, in turn, controlled by antizyme, this regulatory protein appears to be a vital target for pharmacological manipulation. Many of the polyamine analogues down-regulate ODC and polyamine transport, and these effects could be mediated by enhanced antizyme synthesis. It is surprising, therefore, that despite the many polyamine analogues synthesized and studied, there has been no systematic assessment of their efficiency in inducing antizyme synthesis. In the present study, we present the first direct evidence that many polyamine analogues are capable of inducing antizyme. Further, we show that a general correlation exists between the ability of a compound to induce antizyme and its potency in inhibiting the growth of HTC cells in tissue culture.

EXPERIMENTAL

Synthetic polyamine analogues and oligoamines

Spermine analogues BENSPM, SL-11047, SL-11038, SL-11043, SL-11044 and SL-11105 were prepared as described previously [27]. Homospermine analogues bisethylhomospermine (BEHSPM), SL-11098, SL-11102, SL-11103, SL-11108 and SL-11114 were prepared as described previously [28]. BE-4-4-4-4 analogues (pentamines) SL-11122, SL-11123, SL-11127 and SL-11141 were prepared as described elsewhere [29]. Oligoamines SL-11158, SL-11160, SL-11159, SL-11207, SL-11179, SL-11144 and SL-11172 were prepared according to the general procedure described below for the synthesis of SL-11144 and SL-11159.

Synthesis of oligoamines (general procedure)

Preparation of SL-11144 [3,8,13,18,23,28,33,38,43,48-deca-aza-(*trans*-25)-pentacontene]

Pentamide 1 was obtained by the procedure described elsewhere [29] for the analogous triamide. Solutions of pentamide 1 (3 mmol) and of diester 2 (1.5 mmol) [27] in dimethylformamide (20 ml) were kept under an N₂ atmosphere at 5 °C, during which NaH (3.6 mmol; a 60% dispersion in oil was used) was slowly added. The stirred mixture was kept at 22 °C overnight, then the solvent was evaporated *in vacuo*, and the residue was partitioned between chloroform and conc. NH₄Cl solution. The organic layer was separated, washed twice with the NH₄Cl solution and evaporated to dryness. The residue was purified by flash chromatography on a silica-gel column (EM Science, Darmstadt, Germany; 230–400 mesh ASTM) using n-hexane/ethyl acetate (3:2, v/v) as the eluent. *trans*-Decamide 3 was obtained in 72% of the yield. ¹H-NMR (CDCl₃), δ 0.95 (t, 3H), 1.10–1.40 (br, 32H), 2.30 (s, 30H), 2.40–2.60 (br, 60H), 3.65 (d, 4H), 5.40 (t, 2H), 6.95 (s, 20H). Matrix-assisted laser-desorption/ionization MS (*m/z*): calculated, 2533.59 (*M*⁺); found, 2566.80 (*M*⁺ + Na).

A solution containing 1.27 g (0.50 mmol) of pentamide 3, 3.5 g (37 mmol) of phenol and 25 ml of 33% HBr in acetic acid in 25 ml CH₂Cl₂ was kept at 25 °C for 18 h. Water (25 ml) was then added, the aqueous layer was separated and repeatedly extracted with CH₂Cl₂ (3 × 10 ml), and the aqueous solution was evaporated to dryness. The residue was dissolved in a 50% KOH solution (8 ml), and the oligoamine was extracted into CH₂Cl₂

(4 × 10 ml). After the organic solvent was evaporated to dryness, the residue was dissolved in ethanol, and decahydrochloride was precipitated by careful addition of conc. HCl; 540 mg (90%) of SL-11144 decahydrochloride was obtained. ¹H-NMR (²H₂O), δ 1.30 (t, 6H), 1.60–1.90 (br, 32H), 2.95–3.25 (br, 36H), 3.75 (d, 4H), 6.05 (t, 2H); ¹³C-NMR (²H₂O), δ 13.28, 25.57, 45.66, 49.00, 49.13, 49.64, 50.86, 131.15. Electrospray ionization MS (*m/z*): calculated, 711.22 (free base); found, 712.22 (*M*⁺ + H), 747.80 (*M*⁺ + HCl), 356.40 (*M*²⁺ + H) (see Scheme 1).

Preparation of SL-11159 (3,8,13,18,23,28,33,38,43,48-deca-azapentacontene)

A solution of 200 mg of SL-11144 decahydrochloride in 10 ml of water was reduced with hydrogen over 20 mg of PtO₂ at 34.5 kPa for 2 h. The catalyst was separated and the solution was evaporated to dryness *in vacuo*, then the residue was crystallized from water/ethanol solution; SL-11159 decahydrochloride was thus obtained in 85% of the yield (170 mg); m.p. > 250 °C (dec). ¹H-NMR (²H₂O), δ 1.30 (t, 6H), 1.60–1.90 (br, 28H), 2.90–3.25 (br, 32H). ¹³C-NMR (²H₂O), δ 13.27, 25.60, 45.68, 49.03, 49.70. Electrospray ionization MS (*m/z*): calculated, 570.99 (free base); found, 572.0 (*M*⁺ + H), 286.46 (*M*²⁺ + H) (see Scheme 1).

Cell culture

Rat hepatomal (HTC) cells were grown in monolayer and suspension cultures in Swim's 77 medium containing 10% (v/v) calf serum as described previously [21]. Where indicated, horse serum was substituted for calf serum in order to minimize the exogenous spermine and spermidine degradations resulting from the amine oxidases of calf serum. Since HTC cells do not grow quite as well on horse serum, all growth rate experiments utilized the normal calf serum. Previous studies have shown that bisethylpolyamine analogues, used in the present study, are effective for at least 6 days, even in media containing calf serum [27].

Antizyme assay

Cell pellets were homogenized by brief sonication in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.0 mM dithiothreitol and 0.2 mM EDTA. Samples of the cell homogenate were mixed with 0.5 unit of ODC produced in the HTC cells in 0.1 ml of 0.02 M 3-(*N*-morpholino)-2-hydroxypropanesulphonic acid buffer (pH 7.2) containing 0.5 mM EDTA, 1.0 mM dithiothreitol and 0.02% Brij-35. After 15 min on ice, an additional amount of the 3-(*N*-morpholino)-2-hydroxypropanesulphonic acid buffer was added to a total volume of 0.175 ml, and the mixture was assayed for ODC activity as described previously [30]. This assay measures the release of ¹⁴CO₂ from L-[1-¹⁴C]ornithine. One unit of ODC is defined as the amount of enzyme required for releasing 1 nmol of ¹⁴CO₂/h. Antizyme activity was determined by measuring the loss of ODC activity attributed to antizyme addition. As indicated previously [30], inhibition of ODC activity by up to 75% was linear with respect to the amount of antizyme added. One unit of antizyme activity is defined as the amount of enzyme required for inhibiting 1 unit of ODC.

Immunodetection of antizyme protein

Affinity-purified polyclonal rabbit antibody specific for antizyme was prepared by using an antizyme fusion protein expressed in bacteria as described previously [21]. Aliquots of frozen cell

Scheme 1 Synthesis of oligoamines SL-11144 and SL-11159

pellets suspended in SDS sample buffer were chromatographed on 12% polyacrylamide gels and blotted to nitrocellulose membranes, and the antizyme bands were visualized by immunodetection as described previously [31]. To evaluate the relative band intensities, the blots were scanned using Photoshop 4.0 and an Envisions 8100 scanner. The images were then analysed using ImageCalc program of van de Lest et al. [32]. Although three antizyme isoforms have been identified in mammalian cells [33,34], only two are likely to be expressed in this cell line. Of these, we have only found ODC antizyme 1 to be expressed to a significant level in HTC cells, and this antizyme isoform accounts for all the bands detected by this immunodetection procedure.

HTC cell viability and activity

The effects of polyamine analogues on cell growth and activity were determined after the exposure of a set length by use of the indicator dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). As described by Morgan [35], this assay relies on both cell number and metabolic activity. Cells grown on 96-well plates were incubated with MTT as described previously [27], and the product was evaluated using the 555 nm filter on a Bio-Rad EIA spectrophotometer.

Polyamine assays

The levels of the polyamines and the polyamine analogues (except for the oligoamines) were determined by HPLC analysis of dansylated derivatives. Cell pellets containing approx. 1 mg of protein were extracted with 5% HClO₄ containing an internal standard of 1,7-heptanediamine, and they were subsequently reacted with dansyl chloride. The extracts were then separated on a small C-18 reversed-phase column, using a linear gradient of 60–100% acetonitrile/heptanesulphonate (10 mM, pH 3.4), essentially by the method of Minocha et al. [36].

RESULTS

Induction of antizyme by spermine

To investigate the antizyme induction by polyamine analogues in intact cells, it was first necessary to define the parameters of

antizyme generation by the native polyamines. In the present study, rat hepatoma HTC cells were used, because of our extensive experience with polyamine transport as well as antizyme induction and processing in this cell line. Since spermine is considered to be the most effective of the natural polyamines in inducing the +1 translational frameshift required for antizyme production *in vitro*, it was chosen as the benchmark for these cellular studies. Suspension cultures of HTC cells exposed to 10 μ M spermine demonstrated a rapid, but limited, increase in free antizyme activity. As shown in Figure 1(A), there was no further increase in free antizyme activity after approx. 3 h. Since some authors [37,38] have suggested that measured antizyme activity may be decreased by small amounts of antizyme bound to either ODC or the antizyme inhibitor, the levels of antizyme protein were also evaluated by Western blotting/immunodetection techniques. As shown in Figure 1(B), all antizyme bands increased during the 3 h exposure to spermine. Furthermore, quantification of such antizyme protein bands by densitometric scans showed changes that closely reproduced those observed in free antizyme activity.

The relationship between spermine concentration in the media and the resulting antizyme induction is depicted in Figures 1(C) and 1(D). As above, the changes in apparent antizyme protein correlated closely with the measured free antizyme activity. Half-maximal antizyme was induced by 0.2–0.3 μ M of spermine. Although we have not established the affinity of spermine for the HTC polyamine transporter, this value is in reasonable agreement with the K_t values we have previously reported for spermidine uptake into HTC cells [39]. Based on these experiments, a 3 h exposure to 10 μ M of the compound was used as a standard treatment for comparison of the analogues with spermine.

Induction of antizyme by polyamine analogues

Since spermine and spermidine are more effective than putrescine in *in vitro* induction of antizyme, the present study focused on analogues that were structurally similar to the higher polyamines. Almost all analogues in this comparison group are *N*-bisethyl derivatives substituted at the primary amino residues, because this modification appears to stabilize the compounds against

Figure 1 Antizyme (Az) induction by spermine

Before antizyme induction, HTC cells were suspended in fresh medium containing 10% horse serum and incubated in a stirred flask for 20 h. (**A, B**) Spermine ($10 \mu\text{M}$) was added to the medium and cell samples were withdrawn at the indicated times for assaying antizyme activity (**A**) and antizyme protein (**B**). The experiment shown is representative of six such time-course studies. The antizyme assay values shown in (**A**) are the means of two identical assays. (**B**) Two identical samples from the first 3 h of spermine exposure were analysed for antizyme protein by immunodetection of Western blots. (**C, D**) Pretreated HTC cultures were divided into eight small suspension cultures to which different levels of spermine were added as indicated. After a 3 h incubation, samples were withdrawn and analysed for antizyme activity (**C**) and antizyme protein (**D**). Antizyme activity values in (**C**) are the means $\pm$ S.D. for five distinct replicates of the above titration experiment. Western blots were performed on all of the titration experiments and a typical comparison of the antizyme protein levels is shown in (**D**).

degradation by cellular and extracellular amine oxidases and it does not diminish uptake by the polyamine transport system [2]. As shown in Figure 2, the polyamine analogues chosen for the present study can be grouped into four structural types. The first group consists of spermine analogues, which are tetramines where the central nitrogen atoms are linked by four carbon units and the central nitrogen atoms are linked to the external nitrogen atoms by three carbon chains (the exception being BENSPM, where the central chain is a three-carbon group). The central butane residue, which has free rotation in spermine, is restricted by a double bond (as in SL-11047) or by the alicyclic residues that confer *cis-trans* isomerism on these analogues [12,27]. The second group consists of homospermine analogues, which are tetramines where all the amino residues are linked by butane chains. The free rotation of the aliphatic bonds was restricted by alicyclic residues (as in SL-11098), or by double bonds that confer a geometric isomerism on the analogues. In one case (SL-11103), the central butane residue was made rigid by introduction of a triple bond [28]. The third group consists of BE-4-4-4-4 analogues (pentamines), where free rotation was also restricted in several cases by the introduction of *cis*-double bonds [29]. Finally, the fourth group consists of oligoamines, a new type of analogue comprising octamines, decamines and dodecamines, several of which are conformationally restricted.

The ability of these compounds to stimulate antizyme when added to cultures of HTC cells is shown in Figure 3. All experiments were performed both with and without the addition of $10 \mu\text{M}$ of spermine controls, and the antizyme induced by

analogues was compared with that induced by the spermine controls. Surprisingly, almost all the analogues stimulated at least some level of antizyme synthesis. Furthermore, within each structural group there were compounds that induced as much antizyme synthesis as spermine. Although many members of the first three structural groups were either ineffective or substantially less effective than spermine, most of the members of the fourth group were substantially better than spermine in inducing antizyme.

Since antizyme induction was tested under the standardized conditions of $10 \mu\text{M}$ exposure for 3 h, the variability observed in response to the different analogues might be due to the use of sub-optimal conditions for a given test compound. This possibility was examined using compounds representative of each of the structural groups. As shown in Figure 4, regardless of whether a compound induced antizyme poorly (SL-11043) or very well (SL-11144), all the compounds tested (including four compounds not shown here) exhibited half their maximal response at about the same concentration, i.e. $0.6\text{--}0.8 \mu\text{M}$. Clearly, the $10 \mu\text{M}$ concentration used in Figure 3 was sufficient to give a maximal antizyme response for each of the analogues. Similarly, the experiments shown in Figure 5 demonstrate that a 3 h exposure was sufficient for optimal antizyme response. In Figure 5(A), the time course of antizyme induction is compared for compounds that are not as effective as spermine in inducing antizyme. In Figure 5(B), the time course of antizyme induction is shown for compounds that induce much more antizyme than spermine.

Name	Structure	IC ₅₀ (μ M)
Spermidine	<chem>NCCCNCCCN</chem>	---
Spermine	<chem>NCCCNCCCNCCCN</chem>	---
Spermine analogues		
BENSPM	<chem>CCNCCCNCCCNCCCN</chem>	0.40
SL-11047	<chem>CCNCCCNCCCNCCCN</chem>	0.35
SL-11038	<chem>CCNCCCNCCCNCCCN</chem>	0.10
SL-11043	<chem>CCNCCCNCCCNCCCN</chem>	1.2
SL-11044	<chem>CCNCCCNCCCNCCCN</chem>	0.37
SL-11105	<chem>CCNCCCNCCCNCCCN</chem>	16
Homospermine analogues		
BEHSPM	<chem>CCNCCCNCCCNCCCN</chem>	0.22
SL-11098	<chem>CCNCCCNCCCNCCCN</chem>	0.09
SL-11102	<chem>CCNCCCNCCCNCCCN</chem>	0.37
SL-11103	<chem>CCNCCCNCCCNCCCN</chem>	0.87
SL-11108	<chem>CCNCCCNCCCNCCCN</chem>	0.33
SL-11114	<chem>CCNCCCNCCCNCCCN</chem>	0.21
Pentamines		
BE 4-4-4-4	<chem>CCNCCCNCCCNCCCN</chem>	0.14
SL-11122	<chem>CCNCCCNCCCNCCCN</chem>	0.15
SL-11123	<chem>CCNCCCNCCCNCCCN</chem>	0.21
SL-11127	<chem>CCNCCCNCCCNCCCN</chem>	0.13
SL-11141	<chem>CCNCCCNCCCNCCCN</chem>	1.7
Oligoamines		
SL-11158	<chem>CCNCCCNCCCNCCCN</chem>	0.14
SL-11160	<chem>CCNCCCNCCCNCCCN</chem>	0.14
SL-11159	<chem>CCNCCCNCCCNCCCN</chem>	0.073
SL-11207	<chem>CCNCCCNCCCNCCCN</chem>	0.30
SL-11179	<chem>CCNCCCNCCCNCCCN</chem>	0.32
SL-11144	<chem>CCNCCCNCCCNCCCN</chem>	0.23
SL-11172	<chem>CCNCCCNCCCNCCCN</chem>	0.12

Figure 3 Antizyme-inducing potential of the polyamine analogues

HTC cells were suspended in a fresh medium containing 10% horse serum and incubated in a stirred flask for 20 h. Cultures were split into multiple small stirred flasks and the indicated analogues were added to 10 μ M spermine. After a 3 h incubation, cell samples were withdrawn and analysed, as above, for both antizyme activity and antizyme protein. The benchmark response in each experiment was the antizyme level produced by 10 μ M of spermine minus any present in a no-add control. Error bars indicate an S.D. with $n \geq 3$.

Figure 2 Structural representations of the compounds used in the present study and their toxicity in HTC cells

The polyamine analogues, all of which are ethylated on their terminal nitrogens, are divided into different groups based on their structural similarities. The first set differs from bisethylspermine by structural modifications in the central butyl moiety. The second set consists of variations of BEHSPM. Compounds of the third set are all related to BE-4-4-4-4, whereas those of the last set are larger oligoamines with 8–12 amino groups. The sensitivity of HTC cells to these analogues was tested by using an MTT assay [27,35]. HTC cells were seeded in 96-well plates at 250 cells/well. After incubating for 24 h, analogues were added to achieve concentrations ranging from 0.05 to 20 μ M. After 6 days, cell growth and viability were assessed by an MTT assay, and the analogue concentration required to decrease the growth to half-maximal was calculated as IC₅₀.

Figure 4 Titration of analogue concentrations required for antizyme induction

Analogues that induce much more antizyme than spermine (▼, SL-11144), as much antizyme as spermine (●, SL-11047) or much less antizyme than spermine (□, SL-11043; ▲, SL-11102) were titrated as shown in Figure 1(C). ●, Antizyme induced by spermine. All titration curves were normalized to 100% of the antizyme induced by 10 μ M. The values shown for spermine are the means for six independent experiments and the values shown for each of the analogues are for two independent experiments.

Previous studies have suggested that many of the bisethyl-spermine analogues utilize the polyamine transport system for cellular uptake [17,40]. The similar titration curves and time courses of antizyme induction among this collection of analogues are consistent with common use of the polyamine transporter. Two additional lines of evidence confirm this point. First, tested analogues, including the very effective antizyme inducer SL-11144, were found to be ineffective in stimulating antizyme production in PATD cells, a sub-clone of HTC that is defective in polyamine transport (results not shown) [39]. Secondly, the analogues accumulated to much higher levels in those cells where the activity of the polyamine transport system had been enhanced by the inhibition of polyamine synthesis with DFMO. In the present study, HTC cells were exposed to 4 mM of DFMO for 48 h, which greatly decreases cellular putrescine and spermidine levels and increases the activity of the polyamine transporter by 5–6-fold [21]. These cultures, and untreated controls, were then exposed to 10 μ M levels of a representative group of analogues (SL-11047, SL-11038, SL-11043, SL-11108, SL-11102), and the cellular levels of these compounds were determined after 3 h. Whereas the untreated control cultures incorporated the analogues between 1.0 and 1.9 nmol/mg of protein, the DFMO-treated cultures incorporated almost 10-fold more, at 8–16 nmol/mg of protein. By comparison, the spermine levels in these cultures were 10–12 and 8–9 nmol/mg of protein respectively. Interestingly, despite this large difference in cellular analogue levels, there was essentially no difference in the amount of antizyme protein produced and, in general, the relative amounts of antizyme produced by the different analogues were

Figure 5 Comparison of time-dependency of antizyme induction

(A) Exposure of HTC cells to compounds inducing lower levels of antizyme: ◆, SL-11102; ▼, SL-11103; ■, SL-11123. (B) Exposure of HTC cells to compounds inducing higher levels of antizyme: ■, SL-11159; ▼, SL-11144; ◆, SL-11160. (A, B) Exposure of HTC cells to 10 μ M spermine (●). After exposure, the samples were withdrawn and assayed as shown in Figure 1(A). At least two time-course studies such as these were performed on each of the polyamine analogues listed in Figure 2. Data from two such experiments are compared in (A, B) above. In all cases, antizyme increase was essentially completed by 3 h.

maintained. Considering these results, it is clear that the analogues rely on the polyamine transporter and, similar to the native polyamines, cellular uptake is controlled by feedback inhibition mediated by antizyme [21].

Antizyme is a labile protein in HTC cells (half-life $t_{1/2} \approx 75$ min); changes in its half-life, associated with variations in the osmotic potential of culture media, have been shown to control the steady-state level of this protein induced by polyamines [31]. Similar antizyme half-life studies were conducted to determine whether the elevated antizyme level resulting from SL-11144, as compared with spermine, was due to analogue-induced protein stabilization. After a 3 h induction with 10 μ M of either SL-11144 or spermine, 0.2 mM of cycloheximide was added to the cultures, and the cell samples were subsequently extracted and assayed for both activity and antizyme protein. Although

Figure 6 Correlation between the ability to induce antizyme and the propensity to inhibit cell growth and viability

HTC cells were seeded in 96-well plates at 250 cells/well. Analogues (5 μ M) were added after 3 days of incubation, and cell growth and viability were determined 3 days later by using an MTT assay. Some wells received either no addition (controls) or addition of 5 mM DFMO (complete inhibition of polyamine synthesis). Antizyme-inducing potential was derived from the analysis shown in Figure 3. Means $\pm$ S.D. ($n \geq 8$).

there was an approx. 2-fold difference in initial antizyme level, there was no difference observed in half-life of antizyme (results not shown).

Correlation between antizyme induction ability and cell growth inhibition

If a polyamine analogue does not functionally replace native polyamines but does stimulate the formation of antizyme, then the resulting decrease in cellular polyamine levels should inhibit cell growth. Furthermore, there should be a correlation between the propensity of a compound to induce antizyme and the degree of growth inhibition elicited. Two different growth studies were performed on these compounds. In the first study, HTC cells were seeded in 96-well plates at 250 cells/well. After 1 day of growth, different levels of a test compound were added to the medium, and cell growth was determined after 6 days using an MTT assay. All of the bisethylpolyamine compounds tested were found to inhibit growth. IC_{50} , which is the concentration of each compound required for inhibiting cell proliferation to 50% of control values, is shown in Figure 2. With the exception of SL-11105, all IC_{50} values were close to each other over the range of 0.1–1 μ M, which is consistent with the observation that these compounds have been found to enter cells by a common mechanism, the polyamine transporter, for which they have similar affinity. Thus exposure of HTC cells to 5 μ M levels of any of these compounds (except SL-11105) is required for eliciting a maximal effect. After 6 days of exposure to this level of the analogues, the number of cells and the activities were very low, routinely < 10% of the untreated controls; hence, it was difficult to detect any differential responses to the test compounds.

To assess the difference in the cellular responses to a saturating dose (5 μ M) of the various analogues, HTC cells were cultured for 3 days before the addition of analogues, and then growth was evaluated after 3 days of exposure by an MTT assay (Figure 6). Control cultures either received no addition or received the

addition of 5 mM DFMO, which effectively inhibits new putrescine synthesis. HTC cells exposed to DFMO generally undergo between 1 to 2 doublings before polyamine depletion prevents further growth. Thus the level of MTT activity after cells are exposed to DFMO for 3 days (arrow in Figure 6) is an indication of the level of cell growth we would expect in the presence of an analogue that induces sufficient antizyme to inhibit completely polyamine synthesis. Almost all of the tested analogues did affect cell growth during the 3 days of exposure; however, there was a surprisingly wide variation in response. In the bisethylpolyamine analogues that are structurally similar to spermine, BEHSPM or BE-4-4-4-4, the decrease in cell growth and activity ranged from 0 to over 50%, with the most effective antizyme inducers producing the greatest inhibition. However, even the analogues that are equivalent to spermine in antizyme induction were not as effective as the ODC inhibitor DFMO in slowing cell growth. In contrast with the more spermine-like analogues, the longer oligoamine analogues appeared to be very potent. Some members of this group induced immediate cytostasis, as opposed to the delayed growth inhibition generally associated with polyamine deprivation. The most potent antizyme-inducing oligoamines (inducing almost twice as much antizyme as spermine) even caused rapid cell death, with complete loss of the initial population of cells within 3 days. Overall, in this type of cell growth study, there appears to be a good correlation between the ability of a compound to induce antizyme and its propensity to inhibit cell growth.

DISCUSSION

The present study shows that several common polyamine analogues, as well as many newly synthesized conformationally restricted oligoamines, stimulate mammalian cells to synthesize antizyme. Induction of this regulatory protein is probably the primary mechanism for the observed down-regulation of putrescine synthesis and polyamine uptake by such polyamine analogues. Consistent with this model, these studies have shown that the compounds that are more effective in stimulating antizyme production are also, in general, more potent in inhibiting cell growth. This correlation suggests that such effectiveness at stimulating antizyme production might be an important consideration in evaluating polyamine analogues for their growth-inhibiting potential.

Antizyme is a labile cell protein with a half-life that does not appear to be controlled by the polyamine or analogue used in its induction. Thus the different levels of antizyme achieved by the various polyamine analogues and oligoamines must be associated with dissimilar rates of synthesis of this protein. Since antizyme mRNA is generally thought to be expressed constitutively [41,42], this implies that the analogues vary in their ability to stimulate the unique +1 translational frameshift that is required for producing antizyme. Clearly, this polyamine-dependent step is not highly specific, as at least some antizyme was induced by almost all the analogues examined. BENSPM, BEHSPM and BE-4-4-4-4, compounds that are structurally very similar to spermine, were all approximately equal to spermine in stimulating antizyme production. Antizyme-inducing potential was negatively affected, however, by certain conformational restrictions, especially those involving three-, four- and five-membered rings or triple bonds in the carbons between the central nitrogens. By contrast, most of the longer oligoamines examined considerably exceeded spermine in their ability to induce antizyme.

The cellular studies presented here do not clearly explain why the various analogues differ in their antizyme-inducing ability. It is possible that the structural analogues differ in their interaction

with the as yet unknown polyamine-dependent reaction promoting the translational frameshift. Direct demonstration of such differential stimulation would best be achieved using an appropriate *in vitro* translational system. The present study assessed the effectiveness of these compounds in intact cells, and the possibility exists that the observed differential induction of antizyme is more closely related to differences in the amount of each analogue accumulated in these cells. Although all the analogues tested are incorporated by the polyamine transport system, their uptake velocities could differ. Thus it may be suggested that some analogues accumulate to higher cellular levels than others, resulting in greater antizyme induction. In opposition to this, the feedback inhibition of polyamine transport by antizyme dictates that the more effective antizyme-inducing compounds should inactivate the transporter more quickly, allowing less cellular accumulation. It is well known that putrescine, which is not as effective as spermine in inducing antizyme, will accumulate to much higher cellular levels than spermine in polyamine-uptake experiments. Also, methylglyoxalbis(guanylhydrazone), which uses the polyamine transporter yet does not stimulate antizyme production at all, is accumulated to very high cellular levels [43]. We have observed, in preliminary studies, some differences between the accumulated cellular levels of analogues; however, it is still not clear whether these cellular levels are controlled by differences in uptake velocity or timing of antizyme-induced transporter inactivation. In addition to uncertainty about the explanation for different cellular levels of the analogues, there also does not appear to be a simple correlation between the cellular levels of analogues and the induction of antizyme. Specifically, while observing analogue incorporation in cultures with and without DFMO pretreatment, we noted almost a 10-fold difference in the incorporated analogue levels, yet the amount of antizyme protein produced was about the same.

In accordance with our initial expectations, a general correlation does appear to exist between the ability of an analogue to induce antizyme and its propensity to inhibit cell growth. However, the present study did show several unexpected results. When polyamine synthesis is completely inhibited, e.g. by DFMO addition, the extant polyamines are usually sufficient to allow an additional 1–2 cell doublings before the complete inhibition of growth. Since many of the analogues used in the present study stimulated antizyme levels equivalent to that induced by excess spermine, we expected that some of these compounds would down-regulate ODC activity, and therefore also putrescine synthesis, just as effectively as the ODC inhibitor DFMO. Thus we expected to observe cell growth inhibition induced by the bisethylspermine-like analogues to be comparable with that observed in DFMO-treated cultures. Contrary to expectations, the effect of these analogues on cell growth was not as rapid as that induced by direct ODC inhibition. The level of antizyme induced by these compounds is probably not sufficient to completely inactivate ODC, or it could be that some of these analogues at least partially replace the functionality of the native polyamines.

Unlike the bisethylspermine analogues, the longer oligoamines induced immediate cessation of cell growth, more rapid even than the cytostasis resulting from DFMO. These oligoamines, which induce antizyme more efficiently than spermine, must be doing more than merely depriving cells of the needed polyamines. Is this rapid inhibition of cell cycling a response to the super-induction of antizyme? In support of this suggestion, there have been recent reports that antizyme may be responsible for targeting, and inducing rapid degradation, of the specific proteins required for normal cell cycling [19,24]. Alternatively, the

observed growth-inhibitory response may not be related to antizyme induction; rather the effect may be attributable to the analogues themselves having a cytotoxic secondary site of action. The MTT assays used in the present study were designed to test the effect of antizyme-inducing compounds on cell growth, and not necessarily on cell killing. In studies evaluating the colony-forming efficiency, it has been noted that many of the polyamine analogues, including both the spermine-like and the longer oligoamines, are far more effective than DFMO. Thus in addition to the induction of antizyme by these compounds, it is clear that secondary sites of action must be considered in evaluating the potential of an analogue to control specifically polyamine homeostasis.

This systematic screen of 24 polyamine analogues has revealed that antizyme induction is not highly specific in its structural requirements. As such, it is probable that at least some antizyme-stimulating analogues will be identified that neither substitute functionally for the polyamines nor exert secondary cytotoxic effects. Such compounds are expected to induce down-regulation of cellular polyamine levels by both decreasing the putrescine synthesis and inhibiting polyamine uptake, resulting in a control of cell growth, which may be of potential clinical relevance. Further studies are needed to determine whether enhanced antizyme levels can be maintained in cultures continuously exposed to appropriate analogues, and what the cellular consequences are of such long-term elevations in antizyme.

This work was supported by the NIH grants CA-82123 and CA-92703 to J. L. A. M.

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Received 1 November 2001/23 April 2002; accepted 25 April 2002

Published as BJ Immediate Publication 25 April 2002, DOI 10.1042/BJ20011612