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Structural Basis for Differential Induction of Spermidine/Spermine N^1-Acetyltransferase Activity by Novel Spermine Analogs

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SUMMARY

The spermine analog N^1,N^{11}-diethylnorspermine (DE-333, also known as DENSPM or BENSPM) is regarded as the most potent known inducer of the polyamine catabolic enzyme, spermidine/spermine N^1-acetyltransferase (SSAT), increasing activity by more than 200- to 1000-fold in certain cell types. The relative ability of a series of eight systematically modified DE-333 analogs to affect SSAT expression was examined in Malme-3M human melanoma cells, one of several cell lines known to be especially responsive to induction of this enzyme. In particular, we examined the relative contribution of induction of enzyme mRNA and prolongation of enzyme half-life to analog-mediated increases in enzyme activity. Induction of enzyme mRNA was most influenced by intra-amine carbon distances; relative effectiveness was found to be proportional to the number of three-carbon units. Stabilization of enzyme was most determined by the terminal *N*-alkyl substituent size; among methyl, ethyl and propyl groups, methyl was least effective. Thus, DE-333, which most potently induces SSAT mRNA and effectively stabilizes SSAT enzyme activity, produces the greatest increase in enzyme activity. Although other contributing mechanisms may be involved, the relative abilities of the various analogs to induce enzyme activity is at least partially attributable to their combined effects on enzyme mRNA and protein half-life. These data reveal the highly sensitive structure-activity relationships that underlie and control spermine analog induction of SSAT activity. Pending further definition of the relationship between SSAT induction and antitumor growth and toxicity *in vivo*, these relationships may be used to optimize therapeutic efficacy.

A number of studies have demonstrated that SPM analogs such as DE-333 [also known as DENSPM or N^1,N^{11}-bis(ethyl)norspermine (BENSPM)] down-regulate polyamine biosynthetic enzyme activities, suppress transport of polyamines and potently up-regulate the catabolic enzyme SSAT. The net result of these effects is depletion of intracellular polyamine pools and a seemingly related inhibition of cell growth. Of these various responses, induction of SSAT is the most differentially affected among the analogs studied to date. The enzyme is rate-limiting in the polyamine back-conversion pathway, whereby, after acetylation by SSAT, SPM is oxidized by polyamine oxidase to spermidine, which, by the same sequence of events, is then converted to putrescine (1, 2). DE-333 is the most potent known inducer of SSAT activity, increasing it by more than 200- to 1000-fold in some cell types (3–5). Because the enzyme is also up-regulated by the natural polyamines, the mechanisms underlying this response have become a subject of interest to several laboratories. On the basis of studies to date, *SSAT* gene expression is known to be regulated at the levels of transcription (6–8), mRNA stability (6, 7), mRNA translation (9, 10) and protein stability (11–14).

Other analogs similar to DE-333 have been synthesized and investigated by others (15–21). Recently, Bergeron *et al.* (22) synthesized a series of DE-333 homologs that were used to investigate the role of intra-amine carbon chain length, terminal nitrogen alkyl group size, and methylene backbone symmetry. In a detailed biological analysis conducted in L1210 murine leukemia cells, Bergeron's group established certain structure-activity correlations between chain length and IC_{50} values and between terminal alkyl substituents and impact on transport potential, ornithine decarboxylase, and (*S*)-adenosylmethionine decarboxylase and SSAT induction.

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ABBREVIATIONS: SPM, spermine; SPD, spermidine; DE-333, N^1,N^{11}-diethylnorspermine; DM-333, N^1,N^{11}-dimethylnorspermine; DiP-333, N^1,N^{11}-di-sopropylnorspermine; DE-343, N^1,N^{12}-diethylspermine; DM-343, N^1,N^{12}-dimethylspermine; DP-343, N^1,N^{12}-di-*n*-propylspermine; DE-443, 3,7,12,17-tetraazanonadecane [N^1,N^{13}-diethyl(aminopropyl)homospermidine]; DE-444, N^1,N^{14}-diethylhomospermine; DM-444, N^1,N^{14}-dimethylhomospermine; SSAT, spermidine/spermine N^1-acetyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Among those analogs that have similar transport characteristics and cellular accumulation in L1210 cells, the greatest heterogeneity in polyamine-related biochemical responses was seen in analog induction of SSAT activity. Because this response is quite variable among cell lines and not particularly robust in L1210 cells, and because extreme induction of SSAT has been associated with cytotoxicity in certain cells (3, 4, 23), it is possible that these analogs may display a different profile of activity in cell lines that induce more SSAT activity.

The study described herein focuses on the effects of eight recently synthesized DE-333 analogs in Malme-3M human melanoma cells, which are known for their responsiveness to analog induction of SSAT activity and are cytotoxically affected by DE-333 (4, 23). The potent response allowed for the detection of subtle structure-function relationships involving mechanisms of SSAT induction. Portions of these findings have recently been published in abstract form (24).

Materials and Methods

Materials. The polyamine analogs were synthesized according to Bergeron *et al.*, (15, 16, 22). Analog nomenclature is defined in Fig. 1. Malme-3M human melanoma cells adapted to grow in RPMI 1640 medium were kindly donated by Dr. R. Shoemaker and colleagues at the National Cancer Tumor Testing Laboratory (Frederick, MD). Human SSAT cDNA (25) was obtained from Dr. R. Casero (Johns Hopkins Oncology Center, Baltimore, MD) and human GAPDH cDNA was obtained from Clontech Laboratories (Palo Alto, CA). The Western blotting kit was purchased from Amersham (Arlington Heights, IL). Duralon nylon membranes used for Northern blotting and PVDF membranes used for Western blotting were purchased from Stratagene (LaJolla, CA) and Millipore Corp.(Bedford, MA), respectively.

Cell culture. Malme-3M human melanoma cells were maintained as monolayer cultures growing in RPMI 1640 medium containing 10% Nu-Serum (Collaborative Research Products, Bedford, MA) as a semidefined serum substitute. Under certain conditions, medium contained 1 mM aminoguanidine to prevent oxidation of polyamines by serum amine oxidases (26). Cells were seeded at 5×10^6 cell/150 mm Petri dish and incubated 24 hr before treatment with polyamine analogs. Cell number was determined electronically.

SSAT activity assays. After various cell treatments, SSAT activity was determined from cellular extracts prepared and measured as described previously (4). Note that the SSAT assay also detects other acetylase activities, which, in basal measurements, may account for up to 70% of the total enzyme activity. However, in analog or polyamine-induced samples, these other activites account for $<5\%$ of the total activity (4).

Intracellular analog pool determinations. Intracellular polyamine analog concentrations were determined on an acid extract of cells using a high performance liquid chromatography system described elsewhere (27).

RNA Northern blotting. Total RNA was extracted with guanidine isothiocyanate and purified by cesium chloride gradient centrifugation as previously described by Fogel-Petrovic *et al.* (6). RNA samples (10 μg) were separated on 1.5% agarose/formaldehyde gels and transferred to membrane. RNA was hybridized (28) to ^{32}P-labeled cDNA encoding human SSAT (25). After exposure to X-ray film, Northern blots were then washed in stripping buffer [2 mM EDTA, pH 8.0, in 0.1% sodium dodecyl sulfate] for 15–20 min at 75° and hybridized again with human GAPDH cDNA. The GAPDH signal was used as an internal control for evaluating RNA loading. Intensity of SSAT mRNA signal on autoradiograms was measured densitometrically and calculated relative to GAPDH mRNA signal and then relative to SSAT mRNA signal in control samples to determine fold-increase.

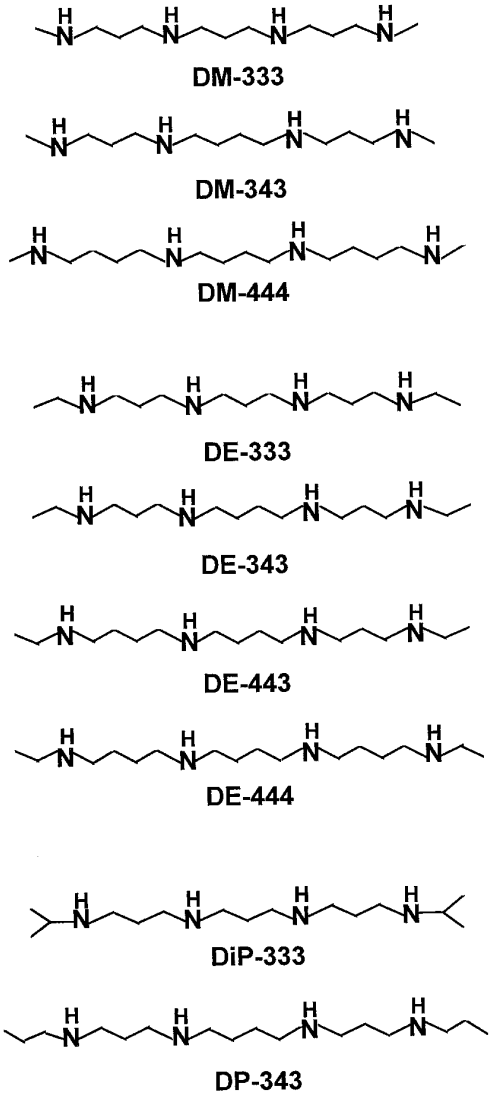

Fig. 1. Structural representations of polyamine analogs used in the present study. Analogs of DE-333 vary according to the length of the intra-amine carbon linkages and the size of the *N*-alkyl substituent.

Western blotting. SSAT protein was detected by immuno-detection with Western blotting using methodologies and an antiserum described in Fogel-Petrovic *et al.* (7).

Protein half-life determination. Malme-3M cells were pretreated for 24 hr with different analogs after which 10 μg/ml cycloheximide was added to block new protein synthesis. Cells were harvested at 0, 6 and 12 hr and SSAT enzyme activity was measured. Treatment of cells with analog plus cycloheximide for longer than 12 hr was found to be toxic.

Results

Analog induction of SSAT activity. To determine analog effects on SSAT activity, Malme-3M cells were treated for 48 hr with 10 μM concentrations of each analog, conditions that were found to be nontoxic with all analogs. Analogs uniformly suppressed ornithine decarboxylase activity.[1] In

[1] D. L. Kramer, M. Fogel-Petrovic, J. Miller, P. Diegelman, J. S. McManis, R. J. Bergeron, and C. W. Porter. Effects of novel spermine analogs on cell cycle progression and apoptosis in MALME-3M human melanoma cells. in preparation.

TABLE 1

Summary of intracellular analog accumulation and relative effect on regulation of SSAT induction

Mean intracellular concentration values were taken from data presented in Kramer et al. (see footnote 1 in text). SSAT mRNA and protein data was taken from Figs. 2 and 3 (and Northern and Western blots not shown), SSAT half-life data was taken from Fig. 4, SSAT protein data was taken from Figs. 2 and 3. Data is presented as mean ± standard deviation of three experiments (mRNA) or at least two experiments (protein). SSAT activity is presented as -fold increase over basal levels (40 pmol/min/mg); because of the contribution of other acetylases to basal levels, however, values for -fold increase underestimate the real induction.

Analog	Intracellular concentration	SSAT mRNA		SSAT half-life	SSAT protein relative to DE-333	SSAT Activity fold increase
		Fold-increase	Relative to DE-333			
	pmol/10^6 cells			*hr*		
DM-333	5825	14 ± 3.5	80	6	46	215
DM-343	5405	8 ± 2.2	37	4	1	10
DM-444	5175	5 ± 2.2	35	6	1	20
DE-333	5965	18 ± 3.6	100	≫12	100	980
DE-343	5535	12 ± 4.4	71	≫12	69	320
DE-443	5220	6 ± 2.1	35	≫12	26	115
DE-444	5165	1.9 ± 0.5	6	≫12	1.8	20
DiP-333	5830	6 ± 2.0	37	≫12	nd[a]	185
DP-343	5675	2.3 ± 0.5	16	14	40	190

[a] n.d., not determined (available analog was found to break down over time).

contrast with this response, induction of SSAT activity was much more varied. Of all the analogs, DE-333 remains the most potent inducer of the enzyme-increasing activity by > 980-fold[2] during a 48-hr period. The four diethyl analogs differentially induced SSAT activity in a manner that correlated very closely with the number of 3-carbon intra-amine units. Thus, the rank order of analogs, according to their ability to induce SSAT activity, was DE-333 > DE-343 > DE-443 > DE-444 (Table 1). Of the three *N*-substituents, the diethyl groups were most effective in inducing the enzyme. For example, DM-333 was much less effective than DE-333. Unlike with the diethyl analogs, there was not a clear ranking among the dimethyl analogs according to number of 3-carbon intra-amine units. Although DM-333 was clearly the most potent, DM-343 was not superior to DM-444. The diisopropyl analog DiP-333 was similar to DM-333 and, unexpectedly, DP-343 was far more effective than DM-343.

Analog accumulation. The differential analog effects on enzyme activity were not related to intracellular analog accumulation. Relative analog accumulation in cells was determined after a 48-hr treatment with 10 μM concentrations of analog (Table 1). Despite substantial differences in structure, all analogs accumulated to very similar intracellular concentrations ranging between 5000 and 6000 pmol/10^6 cells. Interestingly, these levels were nearly equivalent to the summed total of natural polyamines in untreated cells (5810 pmol/10^6 cells; data not shown).

Analog induction of SSAT mRNA. In Malme-3M cells, accumulation of mRNA is known to be a critical component of the enzyme response to polyamine analogs (6–8, 28). SSAT mRNA was assayed in cells treated for 48 hr with 10 μM concentrations of analog and is shown in Figs. 2 and 3. In Table 1, it is expressed as fold-increase and as percent change relative to mRNA induction with DE-333 to facilitate comparisons with SSAT protein data also in Table 1. As with enzyme activity, the fold-increase in SSAT mRNA induction correlated closely with the number of 3-carbon units present in the diethyl analogs (Fig. 2, *left*; Table 1). Thus the rank order of analog induction of SSAT mRNA is DE-333 (18-fold) > DE-343 (12-fold) > DE-443 (6-fold) > DE-444 (2-fold). Because all of these diethyl analogs similarly stabilized SSAT protein, mRNA level seems to be more critical in determining the magnitude of the final enzyme response.

The dimethyl analogs DM-333 and DM-343 are slightly less effective at inducing SSAT mRNA than the diethyl analogs. An inconsistency in the trend was seen in the 444 analogs where DM-444 was ~2-fold better at inducing mRNA than DE-444 (also known as DEHSPM). The dipropyl analog DiP-333 was less effective than DM-333 so that the rank-order of potency for 333 analogs was DE-333 > DM-333 > DP-333 (Fig. 3, *left*) indicating that induction of SSAT mRNA is greatest with the ethyl groups.

Analog induction of SSAT protein. A Western blot analysis (Figs. 2, *right*, and 3, *right*) was performed on samples taken from cells treated with 10 μM concentrations of each analog for 48 hr to compare the levels of SSAT protein

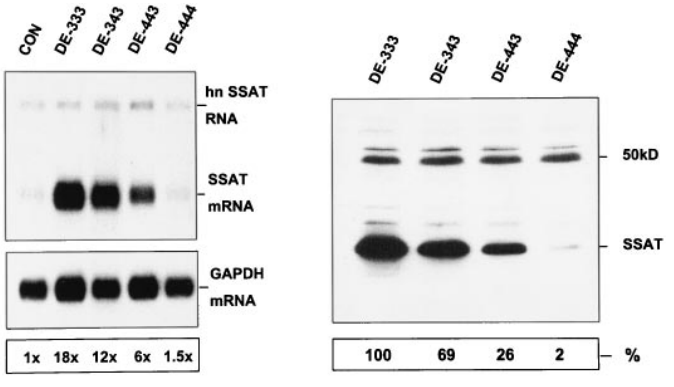

Fig. 2. Representative Northern (*left*) and Western (*right*) blots of SSAT mRNA and protein, respectively, from Malme-3M cells treated for 48 hr with 10 μM diethyl analogs having constant terminal alkyl groups (diethyl) but different intra-amine distances. Fold-increase values at the bottom of the Northern blot (*left*) were obtained by first quantifying SSAT mRNA relative to GAPDH mRNA and then to control SSAT mRNA levels. Western blot data for the diethyl analogs (*right*) is expressed as a percentage of SSAT protein in cells treated with 10 μM DE-333. A 50-kD protein signal was used as a internal control for evaluating protein loading. Data is representative of at least two separate determinations. Data for other analogs are provided in Table 1 (blots not shown).

[2] Because the SSAT enzyme activity assay also measures other acetylases as part of basal activity determinations, the fold-increase after analog induction is certain to be considerably more than that calculated by dividing the induced activity by the basal activity.

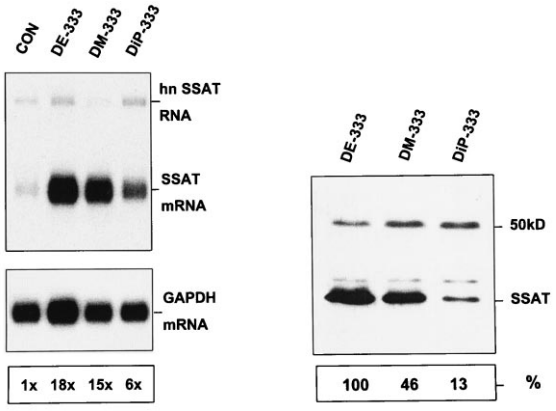

Fig. 3. Representative Northern (*left*) and Western (*right*) blots of SSAT mRNA and protein, respectively, from Malme-3M cells treated for 48 hr with 10 μM concentrations of -333 analogs having constant intra-amine distances (333) but different terminal alkyl groups. Quantification of Northern (*left*) and Western (*right*) blots were carried out as described in Fig. 2. Data is representative of at least two separate determinations. Data for other analogs are provided in Table 1 (blots not shown).

with the differentially induced SSAT activities discussed above. Because the SSAT protein in untreated cells was in very low abundance, the levels in analog-treated sample could be more reliably quantitated relative to DE-333 (100%) induced protein, the highest level detected with any analog. The data in Table 1 show that for each analog, relative SSAT protein correlated relatively well with SSAT activity. Reliable Western blots could not be obtained with DiP-333 because the analog was found to break down with time both in solution and powder form. Thus, data could not be related to earlier results obtained with activity and RNA.

Analog effects on SSAT half-life. We and others have shown that stabilization of enzyme activity (and protein) is involved in SSAT induction by polyamine analogs such as DE-343 (also known as DESPM) and DE-333 (5, 7, 11–14). Uninduced SSAT has been reported to a have a half-life of $<$ 1 hr, which could not be accurately measured in the current studies. Thus, analog effects on half-life are compared relative to one another. As shown in Fig. 4, the four diethyl analogs extend SSAT activity half-life to much more than 12 hr (because of cellular toxicity, cycloheximide could not be reliably used for longer than 12 hr). By contrast, the dimethyl analogs were less effective than diethyl analogs and produced enzyme activity half-lives of 6 hr or less (Table 1). Stabilization of SSAT activity by the dipropyl analogs was similar to that by the diethyl analogs ($>$12 hr). Thus, the *N*-alkyl substituent, rather than the intra-amine distance, seemed more important in determining analog stabilization of SSAT activity and presumably, protein.

Discussion

An original premise for re-examining these analogs was that cells which exhibit an exaggerated SSAT response to analogs may also display structure-activity profiles that are different from those of L1210 cells (22). Whereas the most potent analog (DE-333) induced SSAT activity ~ 15-fold in L1210 cells (at 48 hr), it increased the enzyme $>$980-fold in Malme-3M cells (at 48 hr). In addition, the Malme-3M system offered the opportunity to link analog structure to the various mechanisms underlying SSAT induction and at the same time, to further delineate the nature of this interesting enzyme response.

The various relationships between analog structure and induction of SSAT activity are summarized in graphic form in Fig. 5. In agreement with studies in L1210 cells, (22), the potency of the SSAT response to analogs was significantly influenced by the number of aminopropyl units contained within each analog (i.e., DE-333 $>$ DE-343 $>$ DE-444). This was further defined with DE-344, which in both Malme-3M and L1210 cells induced SSAT activity to levels between those of DE-343 and DE-444. With respect to terminal substituents, we found that diethyl analogs were most effective at inducing SSAT activity, followed by dipropyl and dimethyl analogs. The finding is consistent with substituent mimicry of the acetyl groups of the product of SSAT, N^1-acetylspermine. In addition to confirming these relationships, the bases

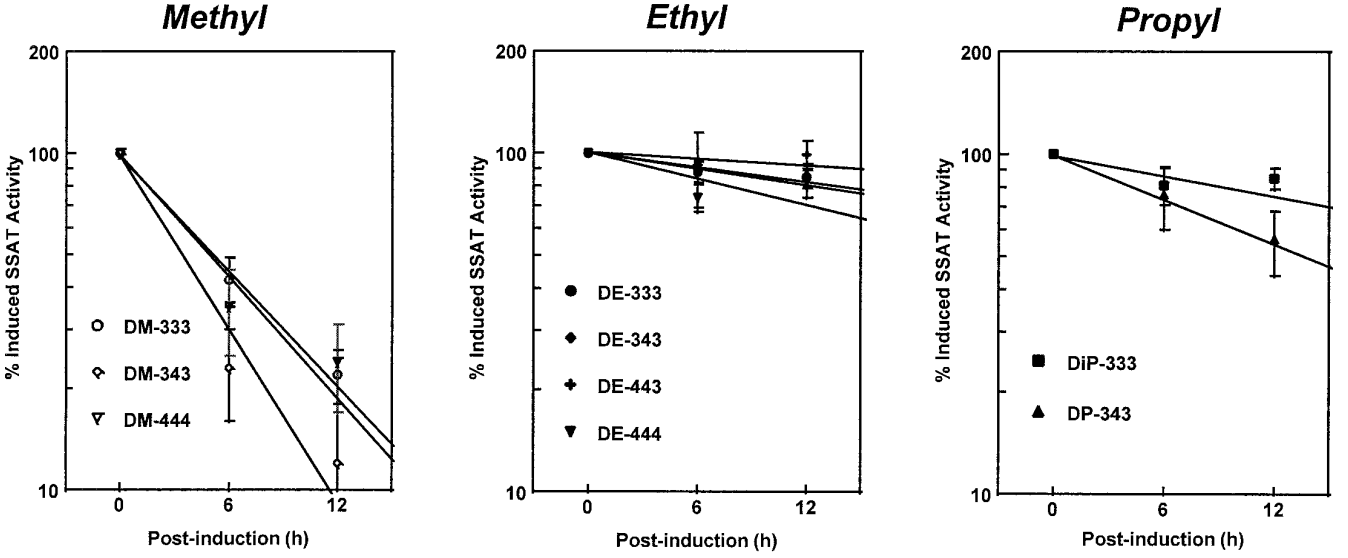

Fig. 4. Half-life determination of SSAT activity in Malme-3M cells treated for 24 hr with 10 μM concentrations of analog and then incubated for 0, 6, 12, hr in fresh media containing 10 μg/ml cycloheximide and 10 μM concentrations of analog. The data represents 3–5 separate determinations for each condition and is expressed as percent of induced SSAT activity using the time zero as 100%.

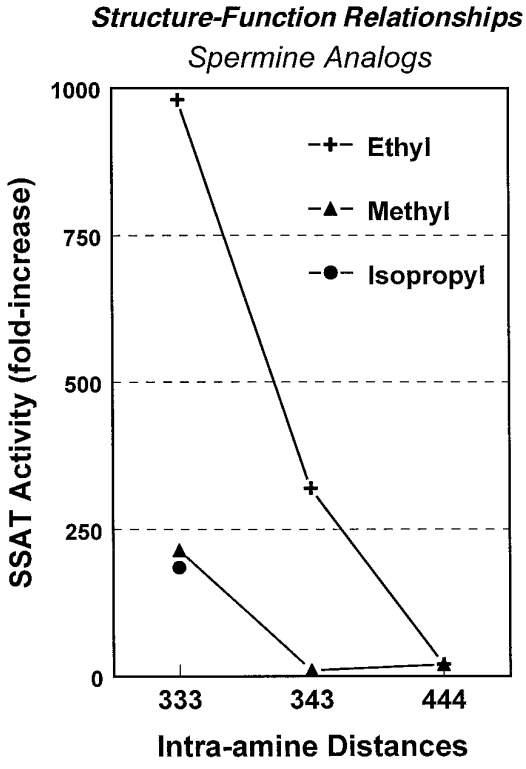

Fig. 5. Graphic representation of the relationships between analog structure and induction of SSAT activity in Malme-3M cells treated with 10 μM concentrations of analog for 48 hr. The *x*-axis represents intra-amine distances for dimethyl (▲), diethyl (✚) and di-isopropyl analogs (●). Only one di-isopropyl analog is represented, DiP-333. This graph was formatted after a figure that appeared in Bergeron *et al.* (32), which depicted analog induction of SSAT activity in L1210 cells. Despite large differences between SSAT inducibility in L1210 and Malme-3M cells, relative analog effects on the response were very similar between the two cell lines.

for them were further defined. Induction of SSAT activity by analogs is known to involve a number of complex mechanisms (6, 7, 10, 14). Included among these are accumulation of SSAT mRNA and stabilization of the enzyme protein, which were studied here. In comparing analogs that have the 333 carbon backbone configuration, we observed that the diethyl and dimethyl analogs induce similar amounts of SSAT mRNA, whereas the dipropyl analogs induced considerably less, despite the fact that all the analogs accumulated to similar intracellular levels. However, when analogs were compared for their ability to prolong the half-life of SSAT activity, the diethyl and dipropyl analogs behaved similarly but the dimethyl analogs were much less effective. Thus, DE-333 is the most effective in inducing SSAT activity because of its combined ability to both induce mRNA and stabilize enzyme activity. DP-333 and DM-333 are less effective but for different reasons; DP-333 is less able to induce mRNA and DM-333 is less able to stabilize enzyme activity. On the basis of these mechanistic distinctions, these analogs may now serve as useful experimental tools for further elucidating this unusually potent enzyme response. At the same time, the exaggerated responses by these analogs allow for the dissection of mechanisms that may also be invoked by the natural polyamines.

The means by which analogs elicit the above SSAT mechanisms is uncertain. In the case of mRNA accumulation in Malme-3M cells, both SSAT mRNA transcription and/or mRNA stabilization have been implicated by both analogs and natural polyamines (6, 7, 10). Such mechanisms could be activated by direct analog interactions at RNA regulatory sites or by analog interaction with a secondary regulatory protein(s) that in turn act at those sites. Alternatively, RNA induction could be the down-stream consequence of analog interference with SSAT function and intracellular polyamine pools. Whatever the mechanism, analogs bearing methyl and ethyl groups can apparently fulfill some binding function(s) more effectively than those bearing propyl groups, because the latter induce much less SSAT mRNA. Stabilization of enzyme activity seems to be caused by a direct analog interaction with the enzyme protein. Coleman *et al.* (14) have shown that the analog DE-343 protects the protein from protease digestion by binding at specific carboxyl-terminal sequences of SSAT. Interestingly, this same region seems to be associated with the enzyme active site. In this present study, we found that diethyl and dipropyl analogs are much more effective than dimethyl analogs at stabilizing SSAT activity, a finding that should probably be evaluated in the Coleman system. This differential analog activity indicates the absence of a unified SSAT response to analogs because, as noted above, the dimethyl are much more effective than the dipropyl analogs at inducing SSAT mRNA. The fact that diethyl analogs activate both mechanisms (i.e., induce SSAT mRNA and stabilize SSAT enzyme protein) seems to explain their superior ability to induce overall enzyme activity.

Examples of how the relative analog effects on various components of the SSAT response can contribute to SSAT activity are shown in Table 2. Selected analogs having differential effects on mRNA accumulation, enzyme half-life and enzyme activity are compared using data distilled from Table 1. Of the five analogs, DE-333 has the greatest impact on both components and, as a consequence, induces the most SSAT activity, whereas DM-343 has the least effect on both components and induces the least activity. Two analogs that have intermediate effects on activity, DE-343 and DM-333, produce substantial increases in SSAT mRNA but differentially affect protein stabilization, which leads to a ~30% difference in enzyme activity. Comparing DE-443 with DM-343 provides the opportunity to examine further the contribution of enzyme stabilization. These two analogs cause more modest increases in SSAT mRNA; however, their differential effects on enzyme half-life seems to account for ~100-fold difference in SSAT activity. The data suggest that under conditions of low mRNA induction, enzyme stabilization seems to have the greater impact on final enzyme activity, but under higher levels of mRNA induction, stabilization of

TABLE 2

Relative contribution of selected analog actions in determining final SSAT activity

Data were selected from Table 1 to focus on the relationships between SSAT mRNA levels, half-life, and activity.

Analog	mRNA (fold increase)	Enzyme $t_{1/2}$	Final activity (fold increase)
		hr	
DE-333	18	≫12	980
DE-343	12	≫12	320
DM-333	14	<6	215
DE-443	6	≫12	115
DM-343	8	4	10

protein exerts less of an effect on final activity. Although other factors may be involved, these comparisons illustrate how analog effects on different components of the SSAT response can contribute in a cumulative manner to final enzyme activity.

Overall, the present findings demonstrate high sensitivity of the SSAT induction and regulation to subtle structural changes in polyamine analogs. Based on impressive antitumor activity in animal systems (29, 30), the analog DE-333 is now nearing completion of Phase I studies targeting solid tumors, such as large cell lung carcinoma and melanoma. There is indication in preclinical models that induction of SSAT may represent a biological response effector (31). By using regulation of SSAT as an example of various molecular events that may be differentially affected by analog structure, it may be possible to optimize clinical indications and/or minimize untoward toxicities by analog design. In addition, analogs that differentially increase SSAT activity are being used in ongoing studies to remove the contribution of enzyme induction to down-stream cellular events, such as those related to cell growth and/or apoptosis.

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