Spermidine/spermine *N*¹-acetyltransferase (SSAT) activity in human small-cell lung carcinoma cells following transfection with a genomic SSAT construct

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Spermidine/spermine *N*¹-acetyltransferase (SSAT) activity is typically highly inducible in non-small-cell lung carcinomas in response to treatment with anti-tumour polyamine analogues, and this induction is associated with subsequent cell death. In contrast, cells of the small-cell lung carcinoma (SCLC) phenotype generally do not respond to these compounds with an increase in SSAT activity, and usually are only moderately affected with respect to growth. The goal of the present study was to produce an SSAT-overexpressing SCLC cell line to further investigate the role of SSAT in response to these anti-tumour analogues. To accomplish this, NCI-H82 SCLC cells were stably transfected with plasmids containing either the SSAT genomic sequence or the corresponding cDNA sequence. Individual clones were selected based on their ability to show induced SSAT activity in response to exposure to a polyamine analogue, and an increase in the steady-state SSAT mRNA level. Cells transfected with the genomic sequence exhibited a

significant increase in basal SSAT mRNA expression, as well as enhanced SSAT activity, intracellular polyamine pool depletion and growth inhibition following treatment with the analogue *N*¹,*N*¹¹-bis(ethyl)norspermine. Cells containing the transfected cDNA also exhibited an increase in the basal SSAT mRNA level, but remained phenotypically similar to vector control cells with respect to their response to analogue exposure. These studies indicate that both the genomic SSAT sequence and polyamine analogue exposure play a role in the transcriptional and post-transcriptional regulation and subsequent induction of SSAT activity in these cells. Furthermore, this is the first production of a cell line capable of SSAT protein induction from a generally unresponsive parent line.

Key words: *N*¹,*N*¹¹-bis(ethyl)norspermine, overexpression, polyamine, post-transcriptional regulation.

INTRODUCTION

Spermidine/spermine *N*¹-acetyltransferase (SSAT) is a rate-limiting enzyme in the polyamine catabolic pathway. This enzyme is essential for maintaining tightly regulated intracellular concentrations of the natural polyamines spermine, spermidine and putrescine. With the goal of exploiting this pathway for therapeutic purposes, polyamine analogues have been developed that possess the ability to disrupt the regulation of this and other enzymes of polyamine metabolism, thereby altering regulation of the naturally occurring polyamines. In human lung carcinoma cells, we have previously demonstrated the existence of a phenotype-specific sensitivity to treatment with one particular class of polyamine analogues, the bis(ethyl)polyamine analogues [1–3]. In sensitive cell lines, represented by the human non-small-cell lung carcinoma (NSCLC) lines, these analogues are thought to utilize cellular regulatory mechanisms to deplete the cell of its natural polyamines, while accumulating the analogue, ultimately resulting in cell death. The relatively insensitive cell lines, represented by human small-cell lung carcinoma (SCLC) lines, show only moderate polyamine depletion and growth inhibition in spite of equal accumulation of analogue. In addition, cells of the NSCLC phenotype exhibit a superinduction of SSAT activity in response to analogue treatment, while SSAT activity levels in the SCLC cell lines remain similar to basal. This association between SSAT superinduction and cytotoxicity has been demonstrated

in several systems, including human melanoma and CHO cells [4,5]. Furthermore, inactivation of the SSAT gene in mouse embryonic stem cells has suggested that growth inhibition by the bis(ethyl)polyamine analogues is not necessarily the result of intracellular polyamine pool depletion arbitrated by SSAT. This cytotoxicity may, rather, be due to an up-regulation of the SSAT/polyamine oxidase pathway, resulting ultimately in the increased production of reactive hydrogen peroxide and aldehydes, and subsequent apoptosis [6].

The regulation of SSAT is complex, and numerous mechanisms are clearly involved in its response to bis(ethyl)polyamine analogue exposure. These include an increase in the transcription and stabilization of mRNA [7,8], an increase in mRNA translation [9,10], and enhanced stabilization of the SSAT protein [11,12]. However, because these analogues alter SSAT expression, much is to be learned about the regulation of the gene without the presence of an inducing agent. In our laboratory and others, overexpression studies aimed at understanding the regulation of SSAT and its role in determining cellular sensitivity to various agents have been hampered by an inability to obtain efficient translation of the transfected SSAT cDNA sequence in human cells [10]. Data using CHO cells stably transfected with the SSAT cDNA sequence have, however, demonstrated a moderate increase in SSAT protein and activity without induction by natural polyamines or polyamine analogues [4]. A successful conditional expression model has also been developed in MCF-7 breast cancer cells using a Tet-off

Abbreviations used: SSAT, spermidine/spermine *N*¹-acetyltransferase; SCLC, small-cell lung carcinoma; NSCLC, non-small-cell lung carcinoma; BENSpm, *N*¹,*N*¹¹-bis(ethyl)norspermine; PRE, polyamine responsive element.

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expression system [13]. It is important to note, however, that both CHO and MCF-7 cell lines are capable of induction and analogue response of SSAT in the absence of a transfected SSAT sequence.

Based on the successful production of SSAT-overexpressing transgenic mice using the genomic sequence of SSAT [14–16], we produced constructs that contained either the genomic sequence or the cDNA sequence covering the entire coding region of SSAT. These constructs were used to stably transfect the analogue-insensitive human SCLC cell line NCI-H82. Results indicate an increase in steady-state SSAT mRNA levels in cells containing either the genomic or the cDNA sequence. However, SSAT protein was detectable only when those cells transfected with the genomic sequence, but not those transfected with the cDNA sequence, were treated subsequently with the polyamine analogue N^1,N^{11} -bis(ethyl)norspermine (BENSpm). Although cells transfected with the corresponding cDNA sequence did demonstrate a moderate increase in steady-state SSAT mRNA, they failed to produce SSAT protein, even in the presence of BENSpm. The data presented in these studies provide evidence suggesting that both an intronic element(s) of the SSAT sequence as well as the polyamine analogue BENSpm are involved in the transcriptional and post-transcriptional regulation of SSAT expression in transfected NCI-H82 cells.

EXPERIMENTAL

Materials

[α - 32 P]dCTP (3000Ci/mmol) was obtained from NEN Life Science Products (Boston, MA, U.S.A.), and [14 C]acetyl-CoA (50–62 Ci/mol) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Trizol reagent, Lipofectin reagent and RPMI 1640 were all purchased from Invitrogen (Carlsbad, CA, U.S.A.). Restriction and modifying enzymes were purchased from Invitrogen and New England Biolabs (Beverly, MA, U.S.A.). ECL[®] Western blotting kit and Hybond ECL nitrocellulose membrane were obtained from Amersham Pharmacia Biotech. Zeta-Probe blotting membrane was purchased from Bio-Rad (Hercules, CA, U.S.A.). Iron-supplemented calf serum, penicillin/streptomycin solution and geneticin were purchased from Gemini Bio-Products (Calabasas, CA, U.S.A.). The polyamine analogue BENSpm was provided by Warner Lambert Parke-Davis (Ann Arbor, MI, U.S.A.). The expression vectors pcDNA3.1(+) and pcDNA3.1(–) were purchased from Invitrogen. The TnT coupled reticulocyte lysate system was purchased from Promega (Madison, WI, U.S.A.).

Cell culture

The human SCLC cell line NCI-H82 was cultured in RPMI 1640 medium supplemented with 9% (v/v) iron-supplemented calf serum, 100 units/ml penicillin and 100 units/ml streptomycin. Cells were counted and viability was assessed using the Trypan Blue exclusion method. For Northern blot, Western blot or SSAT activity analysis, cells were seeded at 5×10^6 cells per 75 cm² flask, in the presence or absence of 10 μ M BENSpm, and incubated for 24 and 48 h at 37 °C and 5% CO₂.

Construction of pSATgen and pSATcDNA plasmids

The 6.2 kb human genomic SSAT sequence [17] was excised from a previously constructed pBS vector and inserted into pcDNA3.1(+) using *Hind*III restriction sites. The 670 bp human

SSAT cDNA sequence was removed from pSATH2 [18] and cloned into pcDNA3.1(–) using *Eco*RI restrictions.

In vitro transcription and translation

The TnT coupled reticulocyte lysate system was used with [α - 35 S]methionine according to the manufacturer's protocol. pSATcDNA was used as template, and the resulting translation product was separated by SDS/12%-PAGE. Results were visualized using Phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Transfection of pSATgen, pSATcDNA or pcDNA3.1 into NCI-H82 cells

Exponentially growing NCI-H82 cells were plated at 3×10^6 cells/well in a six-well plate in serum-free RPMI 1640 medium. Cells were transfected using 20 μ l of Lipofectin reagent with 1.5 μ g of DNA per well, and were incubated for 20 h at 37 °C and 5% CO₂. After 20 h, 5 ml of complete medium was added to each well, and cells were allowed to recover for 48 h. Cells were spun for 5 min at 500 g, aspirated, and resuspended in 10 ml of 0.4 mg/ml G418 selection medium. Cells were maintained in selection medium until a stable population was achieved. Individual colonies were then isolated using serial dilutions.

Analysis of SSAT activity and polyamine content

Cells were plated and treated with BENSpm as described above ('Cell culture'). Cells were then harvested on ice, counted using Trypan Blue, and quick frozen at a final concentration of 2×10^7 cells/ml in SSAT breaking buffer (5 mM Hepes, pH 7.2, 1 mM dithiothreitol). The lysate was assayed for SSAT activity as described previously [1]. Intracellular polyamine and polyamine analogue content was also assayed in this lysate using HPLC by the methods of Kabra et al. [19].

Western blot analysis

Cell extracts were prepared as described above for SSAT activity and separated using SDS/12.5%-PAGE. Proteins were electrotransferred overnight at 20 V on to Hybond nitrocellulose membranes, followed by hybridization with an anti-SSAT antibody [20]. Protein was visualized using ECL Western blotting reagents according to the protocol supplied by the manufacturer, followed by exposure to Kodak X-OMAT film.

Northern blot analysis

Cells were plated and treated as described above ('Cell culture'). Total RNA was extracted from cells using Trizol reagent according to the manufacturer's protocol, separated on 1.5% (w/v) agarose/formaldehyde gels, and transferred to Zeta Probe membranes. The RNA was hybridized with a 32 P-labelled cDNA sequence encoding SSAT according to previously described methods [21]. Blots were stripped and rehybridized with a probe corresponding to 18 S rRNA, to serve as a loading control. The signal intensities of all hybridizations were calculated using Image Quant software with Phosphorimage visualization (Molecular Dynamics).

Growth rate assessment of transfected cells

Cells containing empty vector, pSATgen or pSATcDNA were each seeded in duplicate at 7×10^5 cells per 25 cm² flask. Two flasks were harvested every 24 h for 5 days, and viability was assessed using the Trypan Blue exclusion method.

Polyamine pool and growth responses to BENSpM

Cells were seeded in duplicate at 7×10^5 cells per 25 cm² flask. BENSpM was added at final concentrations of 0, 0.1, 1, 5, 10, and 50 μ M, and cells were incubated for 96 h at 37 °C and 5 % CO₂. Following incubation, cells were collected on ice and counted for viability using the Trypan Blue exclusion method, and an extract was prepared using SSAT breaking buffer as described above. Polyamine pools were quantified using dansyl chloride labelling followed by HPLC, as previously described [19].

Southern blot analysis of SSAT DNA

Genomic DNA was extracted from cloned cells containing the SSAT cDNA sequence or genomic sequence using phenol/chloroform (1 : 1, v/v) and precipitated with ethanol. A portion of 20 μ g of each sample DNA was restriction-digested with BglII, separated on a 1 % (w/v) agarose gel, and transferred to a GeneScreen(+) membrane (NEN) [22]. The membrane was hybridized with a random primer, ³²P-labelled ABS-1 probe [23], resulting in a 2.2 kb fragment representing the endogenous SSAT sequence, and either a 5.0 kb band representing the cDNA sequence or a 6.7 kb band indicative of the genomic construct.

RESULTS

In vitro transcription and translation of pSATcDNA

To verify that the cDNA construct coded for the appropriately sized protein, *in vitro* transcription and translation analysis was performed. The TnT coupled reticulocyte lysate system was used with the pSATcDNA plasmid as a template. This system produced a 21 kDa protein representative of SSAT (results not shown). These results are entirely consistent with our previous use of this system to produce active SSAT protein [24].

Selection of transfectants capable of SSAT protein production and activity

It is important to note that the basal activity of SSAT in H82 cells, as well as in several other SCLC lines, is extremely low and therefore not accurately measurable [1,3,8,18,25,26]. Additionally, previous attempts to induce SSAT activity in SCLC cell lines have not been successful [3]. To establish inducible SSAT activity in H82 cells, we stably transfected the cells with pSATgen, a plasmid containing the entire genomic coding region of the SSAT gene. Ten clones were selected and screened for SSAT activity following a 48 h treatment with 10 μ M BENSpM, an inducer of the enzyme. Of these ten clones, seven exhibited significantly higher SSAT activity following BENSpM treatment as compared with treated empty vector-containing control cells. The clone capable of expressing the highest SSAT activity was chosen for further analyses. The basal SSAT levels of all clones were similar to that of the vector control (Figure 1A). Western blot analysis was consistent with enzyme activity data, with the 21 kDa SSAT band being present only in extracts of cells exposed to BENSpM (Figure 1B). In contrast, multiple transfections and

Figure 1 SSAT activity, protein and mRNA in selected NCI-H82 cell clones

Stable clones containing the empty vector, or the cDNA or genomic SSAT sequence, were treated with 10 μ M BENSpM for 24 and 48 h. Following treatment, cells were harvested for the assay of SSAT activity (A), Western Blot analysis (B) and Northern Blot analysis (C and D). Activity values from a representative experiment are shown in (A) as means, with the ranges for triplicate samples in parentheses. Panel (C) shows total RNA labelled with SSAT cDNA, with the 3.5 kb band representing heteronuclear, pre-processed SSAT RNA, and the 1.4 kb band indicative of fully processed SSAT mRNA. Panel (D) depicts the 18 S rRNA loading control. NT, no treatment.

screening of approximately 100 clones of cells stably transfected with pSATcDNA revealed a lack of cells capable of SSAT protein production following exposure to BENSpM, as previously observed. Therefore clones of cells containing the cDNA sequence were screened by Northern blot for increased SSAT mRNA in both the presence and the absence of 10 μ M BENSpM. Several clones were identified that exhibited significant increases in steady-state SSAT mRNA levels as compared with vector control cells. However, like the control cells, none of these clones demonstrated induction of SSAT protein or activity, even following treatment with BENSpM (Figures 1A and 1B). Therefore the pSATcDNA clone possessing the highest level of basal SSAT mRNA (~4-fold increase) was selected for subsequent studies. Monoclonal, empty vector-containing cells used as controls were selected for G418 resistance.

SSAT mRNA expression in NCI-H82 cells transfected with pSATcDNA compared with pSATgen

Cells were treated with 10 μ M BENSpM for 24 or 48 h prior to RNA extraction. Northern blot analyses revealed a gradual accumulation of SSAT mRNA over the 48 h treatment in all three cell lines, with highest levels in those cells transfected with pSATgen. Northern blot analysis of untreated pSATgen cells revealed a 10-fold increase in the level of 1.4 kb SSAT mRNA over that in untreated control cells. With BENSpM treatment, SSAT mRNA levels in pSATgen cells had increased ~27-fold at 24 h and ~43-fold at 48 h, relative to similarly treated control cells. Heteronuclear 3.5 kb SSAT RNA increased in a manner similar to the 1.4 kb processed mRNA in the pSATgen cells, suggesting enhanced transcription with integration of the genomic

Figure 2 Growth characteristics of transfected NCI-H82 cell lines
Cells were harvested in duplicate each day for 5 days and counted using Trypan Blue exclusion. The y-axis units, N_t/N_0 , represent the ratio of final cell count to initial seeding density. Values represent mean ratios from two experiments, performed in duplicate. Error bars represent the range of values at each point.

SSAT sequence, as well as with polyamine analogue treatment. Cells transfected with pSATcDNA demonstrated a modest 4-fold increase in steady-state levels compared with control cells, and this increase was maintained over the 48 h BENSpM treatment period (Figure 1C).

Growth rate of transfected NCI-H82 cells

Cell viability was assessed daily for 5 days using the Trypan Blue exclusion method. In two separate experiments, cells transfected with pSATgen demonstrated a slight decrease in growth rate when compared with cells transfected with either empty vector or pSATcDNA (Figure 2).

Effects of BENSpM treatment on intracellular polyamine levels and growth inhibition

To determine if increased SSAT activity is associated with intracellular polyamine depletion and growth inhibition in response to BENSpM, cells were treated with increasing concentrations (0.1–50 μ M) of BENSpM for 96 h, at which time cells were harvested for viability counts and lysates were prepared for polyamine analysis (Figure 3). No significant changes were detected in the polyamine pools of untreated cells, regardless of which transfection construct was inserted. Exposure of empty vector- or pSATcDNA-transfected clones to BENSpM treatment resulted in slight growth inhibition, as well as in a moderate decrease in intracellular polyamine pools, consistent with the known ability of BENSpM to down-regulate ornithine decarboxylase activity [1]. In contrast, cells containing the genomic SSAT construct exhibited significantly more growth inhibition, to the point of near-complete cytostasis at 50 μ M BENSpM. Intracellular polyamine concentrations reflected the increase in SSAT-mediated catabolism, with near-complete depletion of all natural polyamines. It should be noted that each of the cell lines examined in these studies readily accumulated BENSpM, and no significant differences were observed in the

Figure 3 Dosage effects of BENSpM treatment on intracellular polyamine pools and cell viability

Cells containing pSATcDNA, pSATgen or empty vector DNA were incubated for 96 h with BENSpM concentrations ranging from 0 to 50 μ M. Cells were treated and harvested for viability assessment by Trypan Blue exclusion, followed by lysate preparation for polyamine pool analysis. Data collected for vector control cells were essentially identical with those for pSATcDNA cells, and are therefore not shown. Polyamine values in (A) represent the means of four determinations, with the range of values in parentheses. Intracellular polyamine concentrations were determined for putrescine (Put), spermidine (Spd), spermine (Spm) and BENSpM. ND refers to levels below detection limits. (B) Dose-dependent effects of BENSpM on the growth of the transfected cell lines. The units of the ordinate, N_t/N_0 , represent the ratio of final cell number to initial seeding density. A value of 1 is therefore indicative of complete growth inhibition, while $N_t/N_0 < 1$ indicates cytotoxicity. Plotted values are means of two separate experiments, performed in duplicate. Error bars represent the range of values observed for each concentration.

amount of analogue accumulated by the differently transfected cell lines.

SSAT DNA copies in transfected NCI-H82 cells

Previous studies have shown no apparent differences in endogenous SSAT copy number between the NCI-H82 cells used in the present experiments and the highly analogue-sensitive NCI-H157 NSCLC cell line [18]. Therefore Southern blotting was carried out on selected stable clones to ensure that integration of plasmid DNA did occur. As expected, clones of cells transfected with pSATcDNA possessed both 2.2 kb and 5.0 kb bands when restricted with *Bgl*III, indicative of the endogenous SSAT gene and the transfected SSAT cDNA respectively. Likewise, cells transfected with pSATgen contained the expected 2.2 kb and 6.7 kb bands, representing endogenous and transfected genomic SSAT sequences respectively. No apparent difference was observed in the prevalence of each exogenous sequence in relation to that of the endogenous SSAT gene (results not shown).

DISCUSSION

Exploitation of the self-regulating nature of the polyamine metabolic pathway has proven to be a rational target for anti-cancer therapy. However, the precise mechanisms through which polyamine analogues elicit their cell-specific cytotoxic responses are not fully understood. To better understand these mechanisms, different strategies have been employed in attempts to overexpress the SSAT gene in several cell lines of different origin. Each of these studies has provided additional insight into the regulatory mechanics, but with substantial limitations. The most frequently encountered problem has been the inability to attain efficient SSAT protein production, in spite of significant overexpression of SSAT mRNA. Successful attempts to increase SSAT protein and activity have been achieved using stable transfection with the human SSAT cDNA in only one human cell line, the human breast cancer cell line MCF-7 [13]. Active protein was also produced in CHO cells transfected with SSAT cDNA [4]. However, it should be noted that, in both cases, the cell types used (MCF-7 and CHO) are capable of inducing their endogenous SSAT mRNA and protein levels in response to analogue exposure [27,28]. In contrast, typical of SCLC cell lines, the NCI-H82 cells used in the present studies are not capable of inducing detectable levels of SSAT protein from their endogenous gene, and are only moderately responsive to bis(ethyl)polyamine analogue exposure with regard to intracellular polyamine regulation and growth inhibition [1,3,25]. However, the results that we present here clearly demonstrate that, following stable integration of an exogenous genomic SSAT sequence, H82 cells not only overexpress SSAT mRNA, but also gain the ability to produce large amounts of active SSAT protein in response to analogue exposure. These results are in contrast with data obtained from H82 cells transfected with the corresponding SSAT cDNA, which also expressed high levels of SSAT mRNA, but exhibited no increase in SSAT activity. These data suggest the possibility of an intronic element(s) in the pSATgen transfection construct whose presence is essential for proper processing of overexpressed SSAT mRNA in the presence of BENSpm.

Importantly, the increased level of SSAT activity in pSATgen cells was associated with an increase in sensitivity to BENSpm. Exposure of pSATgen cells to BENSpm for 96 h resulted in near-complete growth inhibition, accompanied by depletion of putrescine and spermidine to below detectable levels, and near-complete depletion of spermine. Cells containing pSATcDNA or the empty vector also exhibited significant polyamine depletion upon BENSpm treatment. However, the depletion pattern observed in these cells was indicative of the down-regulation of the polyamine biosynthetic enzyme ornithine decarboxylase, which is known to occur in response to BENSpm exposure [1]. These data are in agreement with work by McCloskey and Pegg [29] that provided substantial new evidence that polyamine homeostasis and cellular growth inhibition are indeed a direct result of changes in SSAT regulation, rather than of altered regulation in the polyamine synthetic pathway.

Currently, there is no indication that the *SSAT* gene in NCI-H82 cells or other SCLC cell lines is structurally different from the gene in the more highly responsive cell lines, and no differences have been observed in gene copy number between the two phenotypes [18]. Consequently, the question arises as to why the endogenous gene in NCI-H82 cells does not respond to polyamine analogue exposure. It is known that the polyamine analogues regulate SSAT at the transcriptional level [7,8], and the amount of SSAT mRNA was clearly altered in our pSATgen cells. When exposed to the polyamine analogue, the SSAT mRNA levels of pSATcDNA-containing cells increased to the same extent as

in empty vector-containing cells, while pSATgen-containing cells exhibited a much larger induction of SSAT mRNA. This suggests that the increase in transcription and/or mRNA stabilization induced by the analogue is reliant on a region of the genomic sequence that is absent from the cDNA construct. One such region that should be noted is the polyamine responsive element (PRE) in the 5' untranslated region of the *SSAT* gene. This element was identified in NCI-H157 NSCLC cells, and is necessary for the induction of SSAT mRNA transcription by the polyamine analogues [30]. The PRE has been sequenced in NCI-H82 cells and is completely conserved. Although the PRE is present only in the pSATgen construct, it is unlikely that it is responsible for the observed overexpression, because both constructs are under the transcriptional control of an upstream cytomegalovirus promoter. However, two transcription factors, Nrf-2 and PMF-1, that are required for the analogue-inducible transcription of SSAT via the PRE are expressed only at very low levels in the NCI-H82 cells used in the present studies, even in the presence of BENSpm [30,31]. These data suggest that, in NCI-H82 cells, the *trans*-acting factors, rather than the PRE, may limit the transcriptional regulation of endogenous SSAT in response to BENSpm.

SSAT activity has been implicated in the cell type-specific sensitivity of human cancer cells to various polyamine analogues. The data presented here demonstrate that SSAT activity is responsible for increased sensitivity to a polyamine analogue in a human cell line formerly incapable of significant SSAT production. The pSATgen cells demonstrated near-complete depletion of all three natural polyamines when treated with BENSpm. Although the empty vector- and pSATcDNA-containing cells also exhibited significant polyamine depletion following exposure to BENSpm, it was not as extensive as that observed in the pSATgen-containing cells, and SSAT activity was only marginal in the former. The main phenotypic difference between the cell lines studied here is the induction of SSAT protein. This is consistent with the data of Niiranen et al. [6], which suggest that growth inhibition resulting from exposure to BENSpm may not be due to SSAT-induced polyamine depletion, but rather a result of toxic byproducts of the SSAT/polyamine oxidase pathway. Overall, this system provides an excellent model in which to study important regulatory steps in the expression of SSAT and to determine the precise role of this enzyme in determining drug sensitivity.

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