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Spermine analogs regulated expression of spermidine/spermine N^1-acetyltransferase and its effects on depletion of intracellular polyamine pools in mouse fetal fibroblasts

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Running title: SSAT regulation by spermine analogs

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SYNOPSIS

Spermidine/spermine N^1-acetyltransferase (SSAT), the key enzyme in the catabolism of polyamines, is turned over rapidly and its content in the cell is low. In this study, the regulation of SSAT by spermine analogs, the inducers of the enzyme, was studied in the wild-type mouse fetal fibroblasts expressing endogenous SSAT and in the SSAT-deficient mouse fetal fibroblasts transiently expressing the SSAT-EGFP (enhanced green fluorescent protein) fusion gene. In both cell lines treatments with DENSpm (N^1, N^{11} –bis(ethyl)norspermine), CPENSpm (N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane) and CHENSpm (N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane) led to high, moderate or low induction of SSAT activity, respectively. The detected levels of activities correlated with the presence of SSAT and SSAT-EGFP proteins, the last one localizing both in the cytoplasm and nucleus. Reverse transcriptase -PCR results suggested that the analog-affected regulation of SSAT-EGFP expression occurred mainly at the levels beyond transcription. In the wild-type cells, DENSpm increased the amount of SSAT mRNA and both DENSpm and CHENSpm affected splicing of the SSAT pre-mRNA. Depleted intracellular spermidine and spermine levels inversely correlated with the detected SSAT activities. Interestingly, the analogs also reduced polyamine levels in the SSAT-deficient cells expressing the EGFP control. The results from the present study show that the distinct SSAT regulation by different analogs involves regulatory actions at multiple levels and the spermine analogs, in addition to inducing SSAT, lower intracellular polyamine pools by SSAT-independent mechanisms.

Keywords: spermidine/spermine N^1-acetyltransferase (SSAT), spermine analogs, polyamines, gene expression, enhanced green fluorescent protein (EGFP).

The appreviations used are: SSAT, spermidine/spermine N^1-acetyltranferase; EGFP, enhanced green fluorescent protein; DENSpm, N^1, N^{11} –bis(ethyl)norspermine; CPENSpm, N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane; CHENSpm, N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane.

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INTRODUCTION

The polyamines putrescine, spermidine and spermine are essential in central cellular processes including proliferation and differentiation . The intracellular polyamine pools are tightly regulated by means of biosynthesis, catabolism, uptake and export. In catabolic pathway spermidine/spermine N^1-acetyltransferase (SSAT) catalyzes the acetylation of spermine and spermidine making them suitable substrates for the next enzyme in the pathway, polyamine oxidase, or for the polyamine export machinery. Multiple factors regulate SSAT activity by controlling the gene expression at one or several levels. For example elevated intracellular polyamine concentrations regulate SSAT by increasing gene transcription, stabilizing mRNA, enhancing mRNA translation and increasing protein stability . Similarly, polyamine analogs, the mimics of natural polyamines, can effect SSAT expression at multiple levels. Several types of *N*-alkylated polyamine analogs have been synthetized. They vary in their ability to displace the natural polyamines in the cell as well as to fulfil the biological functions of natural polyamines. In addition, the analogs in general are poor substrates for the polyamine catabolic enzymes. Because of the stability and the functional ability to regulate polyamine metabolic enzymes as well as by the ability to deplete intracellular polyamine pools, the analogs produce a long-term effect which disrupts polyamine metabolism. As a consequence, disturbed cellular functions and cytotoxicity may result. The development of polyamine-based molecules for therapeutic purposes is an ongoing research field and for example the *N*-terminally alkylated polyamine analog N^1, N^{11} –bis(ethyl)norspermine (DENSpm) has been in clinical trials against several human solid tumors . Antiproliferative action of DENSpm seems at least in some cell lines to be connected to the induction of SSAT . DENSpm increases SSAT activity by thousands of folds and effects hnRNA splicing, increases SSAT mRNA level, enhances translation and effects protein stability . In addition to symmetrically substituted alkylpolyamine DENSpm, several unsymmetrically substituted alkylpolyamine analogs such as CPENSpm (N^1-ethyl-N^{11}-((cyclopropyl)methyl)-4,8-diazaundecane) and CHENSpm (N^1-ethyl-N^{11}-((cycloheptyl)methyl)-4,8-diazaundecane) have been studied with the respect to their cytotoxicity and ability to induce SSAT. Like DENSpm, CPENSpm is an effective superinducer and inhibitor of SSAT and has antitumor action *in vitro* . Interestingly, CHENSpm is highly cytotoxic, inhibits SSAT but does not effectively elevate SSAT activity . In the present work, the spermine analog-mediated regulation of SSAT in non-tumour cells was studied. We produced a SSAT-EGFP (enhanced green fluorescent protein) fusion construct to facilitate the use of detectable marker protein. The expression, localization, activity and function of SSAT-EGFP in response to spermine analogs in transiently transfected SSAT-deficient mouse fetal fibroblats (devoid of endogenous SSAT activity) were followed. Addition of relatively stable EGFP at the carboxyl terminus of SSAT abolished neither activity nor low abundance of the SSAT enzyme. For comparison the wild type mouse fetal fibroblasts were used to analyze the regulation of genome-encoded SSAT. The analog-affected changes in SSAT activities of SSAT-EGFP and endogenous SSAT were very similar, although, as expected, the regulation of genome-encoded SSAT by the analogs was shown to be more complex. Interestingly, the levels of intracellular polyamine pools followed the degree of SSAT activity but, as shown in the SSAT-deficient cells, were decreased also by mechanisms independent of the SSAT function.

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EXPERIMENTAL

Materials

DENSpm was synthesized essentially as described by Rehse et al. . CHENSpm and CPENSpm were synthesized as previously described . Generation of mice with disrupted SSAT-gene is described previously .

Syngenic and SSAT-deficient primary fetal fibroblasts were isolated according to the previously described method , except that the fetuses were taken on day 16 of pregnancy. The cells were seeded in six well plates at a density of 3.5 x 10^5 cells/well and grown in DMEM (Sigma) supplemented with 10% FBS and 50 µg/ml of gentamycin at 37°C in 7.5% CO_2 atmosphere. Polyamine analogs were used in 10 µM concentrations.

Production of SSAT-EGFP fusion construct

The open reading frame region of the mouse SSAT gene was amplified from the cloned cDNA with the primers 5'-CTAAAGCTTCAAAAGACGAAAATGGC-3' (forward, the start codon underlined) and 5'-GATGGATCCTCCTCTGCTGCCATTTTTTAG-3' (reverse, the last codon underlined). The PCR product was digested with *Bam*HI and *Hind*III restriction enzymes and ligated in frame with the EGFP gene into the pEGFP-N1 vector (Clonetech). The structure of the constructed plasmid was confirmed by automated sequencing. Plasmid DNAs for transfection were prepared using QIAFilter Maxi kit (Qiagen) following the manufacturer's protocol.

Transfection

Cells were transfected with calcium phosphate as follows. The cells were seeded in six well plates at a density of 3.5 × 10^5 cells/well and incubated for 24 hours. Two micrograms of plasmid DNA was mixed with 40 µl of 0.25 M $CaCl_2$. Then 40 µl of 2 x BBS (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added and the mixture was incubated for 30 minutes at RT. The medium was removed and the cells were washed twice with DMEM. The wells were covered with 1.5 ml of DMEM and 80 µl of plasmid preparation was added into each well. The plates were kept for 2.5 hours in the incubator, after which the medium was removed and the cells were washed as described above. Growth medium without or with the analogs was added and the cells were incubated for 24 hours.

Analysis of ODC and SSAT activities and intracellular polyamine contents

The activities of ODC and SSAT were measured according to the published methods. Natural polyamines were determined by HPLC as described previously . Polyamine analogs were measured using a previously described method .

Western blot analysis

Trypsinized and washed (PBS) cells were lysed in a buffer containing 25 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1% Triton-X-100, 1 mM dithiotreitol, and 1 × Complete EDTA-Free protease inhibitor cocktail (Roche). Samples were centrifuged (15 min, 10 000*g*, +4°C) and protein contents from the supernatants were measured by Bio-Rad Protein Assay kit. Western blot analysis was performed with standard protocols using 30 µg of protein and GFP antibody (BV living colors A.v. peptide antibody, Clonetech) or SSAT antibody. SSAT antibody was affinity-purified from the serums of rabbits injected with the *E. coli*-produced recombinant mouse SSAT protein. Ponceau staining of the membrane and detection of β-actin (Sc1616, Santa Cruz Biotechnology) was used to confirm the equal loading.

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Reverse transcription-PCR

Total RNA from the cells was extracted by TRIzol Reagent (Invitrogen) and treated with Dnase I (Dna-free, Ambion) according to the manufacturer's instructions. One microgram of Dnase-treated RNA was used for first-strand cDNA synthesis using oligo-dT primers and AMV reverse transcriptase (Promega) in a total volume of 25 µl. PCR reaction contained 2 µl of the first-strand cDNA, 1x PCR buffer with $MgCl_2$ (Finnzymes), dNTP mix (200 µM each), 25 pmol forward and reverse primers and 0.5 U DyNAzyme DNA polymerase (Finnzymes) in a total volume of 50 µl. The used primers were as listed below. SSAT: 5'- TACGTCGACATGGCTAAATTTAAGATCCG-3' (forward), 5'-TACGTCGACGATACAGCAACTTGCCAATC-3' (reverse); EGFP: 5'-CCCTGAAGTTCATCTGCAC -3' (forward), 5'-GTTGTACTCCAGCTTGTG-3' (reverse); SSAT-X: 5'-TACGTCGACATGGCTAAATTTAAGATCCG-3' (forward), 5'-TACGTCGACCAATGCTATGTCCTCATTTAT -3' (reverse); 18S RNA: 5'-CTACCACATCCAAGGAAG-5' (forward), 5'-CTCAGCTAAGAGCATCGAG-3' (reverse). The program consisted one cycle of 95°C for 3 min, 24 (18S RNA), 25 (SSAT, EGFP) or 29 (SSAT-X) cycles of 95°C for 30 seconds, 58°C for 40 seconds, 72°C for 40 seconds followed by one cycle of 72°C for 5 min. PCR products were separated on a 0.9% agarose gel and scanned by Typhoon 9400 (GE Healthcare). The images were analyzed by the Image Quant TL program (GE Healthcare).

Microscopy

To examine the fluorescence intensity, the cells were cultured on coverslips. After incubation the cells were washed with PBS and fixed with 4 % paraformaldehyde for 30 min. After that the cells were washed twice for 10 min with PBS. For propidium iodide (PI) staining, the cells were permeabilized with 0.3% Triton-X-100 (Fluka) in PBS for 15 min at room temperature and washed twice for 10 min with PBS. Then the cells were treated with RNase (Boehringer Mannheim Biochemicals) at concentration of 100 µg/ ml in PBS for 20 min at room temperature and washed three times with PBS. The cells were counterstained with PI (Sigma) in PBS at final concentration of 500 ng/ml for 5 minutes and washed three times with PBS. The coverslips were mounted on glass slides and examined under Olympus AX 70 fluorescence microscopy or under Biorad Radiance 2100 confocal laser scanner. An argon ion laser emitting at 488 and 543 was used to excite EGFP and PI respectively. During scanning, a 40 x oil immersion objective was used. Serial optical slices of 1 µm or less were taken.

Flow cytometry analysis

Transfected cells in the six well assay plates were trypsinized, washed (PBS) and suspended in 0.5 ml of PBS. To fix the cells, 0.5 ml of 4 % PFA was added. Proportion of EGFP positive cells was analyzed with flow cytometry (FACScalibur, Becton Dickinson). EGFP expression level was obtained by dividing the mean fluorescence intensity (FL1 channel) of the EGFP positive population by the mean fluorescence intensity of the EGFP negative population.

Determination of SSAT and SSAT-EGFP half-lives

The syngenic and SSAT-deficient cells were seeded in six well plates at a density of 3.5 x 10^5 cells / well. SSAT-decifient cells were transfected with SSAT-EGFP as described above and both the syngenic and transfected cells were grown without or with 10 µM concentration of the analogs for 24 hours. The media was replaced with media containing cycloheximide at final concentration of 10 µg / ml without or with the analogs. Each treatment was repeated three times (experiments). Samples in triplicate were collected from each treatment in four or five timepoints ranging from 0 minutes to 10 hours. SSAT activity of the samples was detected as described above. Average SSAT activity values were calculated for the

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triplicates. Based on these average activity values on different time points a linear regression line was fitted to each experiment within a treatment. The half-lives for SSAT and SSAT-EGFP were estimated from the regression line as: half-life = -0.5 * (*a/b*), where *a* is the intercept and *b* is the regression coefficient of the estimated regression line. For each treatment the mean half-life over the three experiments was calculated.

Statistical analysis

Data are expressed as mean ± *SD* where applicable. GraphPad Prism 4.03 (GraphPad software) software package was used to perform one way ANOVA and Dunnett's multiple comparison test. Linear regression analyses were performed using GLM procedures of the SAS v.9.1 –statistical package (SAS Institute Inc.).

RESULTS

Accumulation of SSAT-EGFP in untreated and spermine analogs treated cells

In order to study the regulation and intracellular localization of SSAT, a CMV promoter driven fusion construct containing the protein coding region of SSAT at the amino terminus of EGFP was made. SSAT-deficient mouse fetal fibroblasts were transfected with the SSAT-EGFP fusion construct or an EGFP control as described in experimental procedures. Cells were grown for 24 hours either without or with 10µM DENSpm, CPENSpm or CHENSpm. During that time, no cytotoxicity was observed (N1/N0 value ≥1). The cells were fixed with 4% paraformaldehyde and studied under fluorescence microscopy. The control cells grown without or with the analogs strongly expressed EGFP and were easily visualized by the microscope. In contrast, the SSAT-EGFP- transfected cells grown without the analogs or treated with CHENSpm contained no detectable signal. However, the intensity of fluorescence in the fusion construct containing cells was enhanced by DENSpm and CPENSpm treatments and could be observed under fluorescence microscopy. The differences in fluorescence intensities of the samples were confirmed by measuring the values for relative fluorescence by flow cytometry. As shown in Figure 1A, the fluorescence of EGFP-expressing control cells was decreased by the analogs, however still remaining several times higher than the fluorescence in SSAT-EGFP-expressing cells. As expected, significant increase in the fluorescence intensity was detected in the SSAT-EGFP-expressing cells grown with DENSpm or CPENSpm compared with the cells without the analog treatment. Fluorescence in the fusion construct-expressing cells grown with CHENSpm was undistinguishable from that of the untreated cells.

To further visualize the presence of fluorescent proteins a western blot analysis was performed from the protein samples of the cells grown under similar conditions as described above. The EGFP control protein was easily detected in high amounts in all EGFP-transfected samples (data not shown). In contrast, in the SSAT-EGFP transfected cells the GFP and SSAT antibodies detected a very low amount of the fusion protein only in the homogenates from the DENSpm-treated cells (data not shown). For comparison, the abundance of SSAT protein in the samples of analog-treated wild-type mouse fetal fibroblasts was analyzed with a western blot. It is known that SSAT is expressed in a very low level and as seen in figure 1B only DENSpm treatment increased the SSAT amount into a detectable level in the experimental set-up and with that particular SSAT antibody used. However, the results supported the view obtained from the cells expressing the SSAT-EGFP fusion protein. Thus, the intensity of the observed fluorescence correlated with the abundance of EGFP and SSAT-EGFP proteins.

Intracellular localization of SSAT-EGFP

To study the intracellular localization of SSAT-EGFP, the same samples which were used for fluorescence microscopy were also analyzed with confocal microscopy. In the case of EGFP control

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expressing cells, the fluorescent protein localized strongly both in the cytoplasm and the nucleus as shown in the merged images of EGFP and propidium iodide -stained nuclei (Fig. 2). Studied analogs had no effect on the localization of EGFP. Because of the higher intensity of the excitation light used in confocal microscope, it was possible to visualize the SSAT-EGFP protein both in untreated and analog-treated cells. In all samples, SSAT-EGFP was localized both in the cytoplasm and the nucleus (Fig. 2). However, occasionally cells in which the analog treatment reduced or fragmented the nuclear localization of SSAT-EGFP were found (data not shown). Whether this reflects the action of SSAT-EGFP or the analog induced changes in the physiology and condition of the treated cells in general remains to be resolved.

Analog-affected SSAT activities and intracellular polyamine levels

Spermine analogs DENSPm, CPENSpm and CHENSpm all act as inhibitors of SSAT *in vitro* . However, only DENSpm and CPENSpm superinduce the enzyme in living cells. To study the function of SSAT-EGFP fusion protein the SSAT activity and the intracellular polyamine levels in transfected cells grown without or with the analogs were measured. As a control, EGFP transfected cells were subjected to similar analysis. SSAT-deficient mouse fetal fibroblasts were used because they lack endogenous SSAT activity. As shown in table 1, the presence of SSAT-EGFP led to nine fold induction in SSAT activity in transfected untreated cells when compared with the cells transfected with EGFP. However, the changes in intracellular polyamine levels were only minor. In contrast, treatments with polyamine analogs caused significant changes in SSAT activities and in intracellular polyamine levels. In DENSpm- treated cells, the activity of SSAT-EGFP was thirty times higher than in the cells grown without the analog and the intracellular spermidine and spermine levels were significantly depleted. CPENSpm and CHENSpm activated SSAT-EGFP by more than sixteen and three folds, respectively, and also significantly decreased spermidine and spermine levels. Also significant differences in the accumulation of putrescine were found: DENSpm depleted and CHENSpm increased the amount of putrescine. The function of SSAT-EGFP protein in transfected cells was also confirmed by the accumulation of N^1-acetylspermidine which is considered as an indicator of SSAT activity. DENSpm treatment caused the highest enzyme activity and protein accumulation of SSAT-EGFP compared with CPENSpm and especially with CHENSpm. Thus, in the case of SSAT-EGFP the observed SSAT activities correlated with the fluorescence and abundance of the fusion protein detected by microscopy, flow cytometry and western blot analysis. Instead, surprising results were obtained from the SSAT-deficient cells transfected only with the EGFP gene. In all samples there were only a low background level of SSAT activity (cells have deficient SSAT gene). However, all tested analogs depleted the intracellular spermidine and spermine levels significantly. In addition, DENSpm and CHENSpm significantly affected the putrescine levels. Interestingly, the changes in polyamine pools in EGFP expressing cells were exactly similar than those in the cells transfected with SSAT-EGFP. Therefore, it seems that all tested analogs induce also a SSAT-independent mechanism to reduce intracellular polyamines in mouse fetal fibroblasts.

In order to compare the SSAT activities of the SSAT-EGFP fusion protein and the endogenous SSAT protein, as well as to study the intracellular polyamine levels, the wild-type fetal fibroblasts were subjected to similar analog treatments as described above. As shown in table 2, the SSAT activities and polyamine pools observed in the wild-type cells with and without the analog treatments were very similar as the ones obtained from the SSAT-EGFP transfected cells deficient of endogenous SSAT.

ODC activity in spermine analog -treated cells

To see how the spermine analogs affect to the production of polyamines, the activity of ornithine decarboxylase (ODC), the key enzyme in the polyamine biosynthetic pathway, was measured in the SSAT-deficient and wild-type mouse fetal fibroblasts. The results are shown in table 3. DENSpm significantly reduced the ODC activity in both cell lines. Interestingly, CPENSpm significantly increased the activity in wild-type cells and significantly decreased the activity in SSAT deficient cells. However,

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CHENSpm did not change the ODC activity in either line. Thus, the decreased intracellular polyamine pools in the analog-treated cells do not totally correlate with the changes observed in biosynthesis.

SSAT mRNA expression in analog treated cells

As the amount of SSAT-EGFP protein was clearly increased by DENSpm and CPENSpm, studies to analyze the steady state level of the fusion construct mRNA were conducted. Reverse transcriptase PCR was run with the primers for the EGFP part, the SSAT part or the junction part of the fusion construct and they all gave similar results. Figure 3 shows the accumulation of EGFP and SSAT-EGFP mRNA-derived signals amplified with EGFP specific primers. Relative optical density (ROD) of the mRNA concentration using target gene / 18S rRNA indicated that CPENSpm slightly increased the transcription of EGFP in the pEGFP-N1 control plasmid-transfected cells. Instead, transcription of SSAT-EGFP fusion construct was not significantly affected by the analog treatments. If anything, DENSpm slightly decreased the amount of SSAT-EGFP mRNA. Thus, the increased amount of SSAT-EGFP protein detected in the DENSpm and CPENSpm exposed cells was not a consequence of enhanced transcription.

It is known that DENSpm stabilizes wild-type SSAT mRNA and also fine-tunes the expression of SSAT by regulated unproductive splicing and translation (RUST) . However, the effects of CPENSpm and CHENSpm have not been published. In order to see how the analogs affect transcription of the genomic SSAT gene and splicing of the SSAT pre-mRNA, RT-PCR from the analog-treated wild-type mouse fetal fibroblasts was performed. The primers detecting the normal SSAT mRNA and the primers amplifying the alternative splice variant, SSAT-X, were used. The results are shown in figure 4. Significantly increased amount of SSAT mRNA was present in the cells treated with DENSpm and CPENSpm. In contrast, CHENSpm did not change the amount of SSAT mRNA pool. In accordance with the previous report , significant decrease in the accumulation of SSAT-X, the unproductive splice variant, in DENSpm treated cells was observed when compared with the amount present in the untreated cells. Interestingly, the unproductive splicing of SSAT pre-mRNA was enhanced by CHENSpm, the analog not able to superinduce SSAT. On the other hand, CPENSpm did not affect the SSAT pre-mRNA splicing compared with the untreated cells. Differences in analog-affected transcriptional regulation between the endogenous SSAT and the transfected SSAT-EGFP fusion construct reflect the important influence of SSAT promoter and the whole genomic sequence, including introns, for the regulation and expression of the gene.

Effects of the analogs on the half-lives of SSAT and SSAT-EGFP proteins

In order to study how the analogs affect the stability of SSAT and SSAT-EGFP the half-lives of SSAT and SSAT-EGFP without or with the analogs were determined by analysing the changes in SSAT activity after protein synthesis inhibitor cycloheximide addition. The results are presented in Table 4. The half-lives of SSAT and SSAT-EGFP were lowest in the untreated cells and were comparable to each other. Both DENSpm and CPENSpm increased the half-lives of SSAT and SSAT-EGFP. The extended half-lives of the proteins were similar compared with each other. However, DENSpm extended the half-lives more intensively than CPENSpm. CHENSpm had only a minor effect on the SSAT half-life, but interestingly, seemed to increase the half-life of SSAT-EGFP quite extensively. Altogether, the results suggest that the wild-type SSAT and SSAT-EGFP have similar half-lives which are extended by spermine analogs.

DISCUSSION

We have generated a SSAT-EGFP fusion construct which is expressed in low amounts but with detectable SSAT activity in transiently transfected SSAT-deficient mouse fetal fibroblasts. The enzyme activity and intracellular accumulation of the fusion protein is significantly enhanced by spermine analogs DENSpm and CPENSpm. The spermine analog CHENSpm only slightly increases the SSAT activity of

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the fusion product. The studied analogs cause similar changes also in the wild-type cells expressing endogenous SSAT. In addition, in all cases the enhanced SSAT activity correlates with the decreased polyamine levels. Thus, the expression of either endogenous SSAT or the transfected SSAT-EGFP can lead to similar cellular changes in mouse fetal fibroblasts. However, when the regulation of SSAT and SSAT-EGFP expression by spermine analogs is analyzed at different levels some differences can be found. We have shown that the SSAT-EGFP mRNA levels in cells grown without or with the analogs are not significantly different (Fig. 3). Thus, transcription of the fusion construct containing only the coding region of SSAT (no 3' or 5' UTRs) is not enhanced by the analogs and hence the increased transcription is not the reason for protein accumulation. It has been reported previously that the transcription of the FLAG-SSAT construct containing only the protein encoding sequence was not increased, but decreased, by DENSpm . On the other hand, in our study a significant increase in the SSAT mRNA level was obtained in the wild-type mouse fetal fibroblasts showing the ability of DENSpm to induce the SSAT transcript production from the genomic SSAT. This also is the case *in vivo* since the amount of SSAT transcript in the liver samples isolated from DENSpm-treated mice is higher than in the samples from untreated mice . In addition, we showed that the splicing of SSAT pre-mRNA is directed towards the variant transcript, SSAT-X, by CHENSpm and as reported previously production of the normal SSAT mRNA is enhanced by DENSpm. Thus, the regulation of genome-encoded SSAT is differentially fine-toned by spermine analogs. Altogether, our findings confirm that the complete regulation of endogenous SSAT gene expression happens at multiple levels and involves the promoter and the exonic and intronic sequences as well as the 3' and 5' UTRs.

Because of the low expression level, the intracellular localization of SSAT has been difficult. Under DENSpm-induced conditions using immunohistochemistry and immunocytochemistry, SSAT has been detected in the cytoplasm of human adenocarcinoma A549 cells and in the cytoplasm, nucleus and mitochondria of the human breast cancer L56Br-C1 cells, respectively . We were not able to detect the SSAT-EGFP under the fluorescent microscopy unless DENSpm or CPENSpm was added to the growth medium. However, confocal microscopy studies facilitated the detection of the fusion protein in all conditions. SSAT-EGFP localized both in the cytoplasm and in the nucleus in untreated and analog treated samples. The other cell organelles were not studied in detail for the presence of SSAT-EGFP. Since EGFP is widely used to determine the subcellular localization of other proteins SSAT-EGFP could offer a tool to follow the intracellular localization of the low abundant SSAT in different types of cells, including non-tumor cells (in normal growth conditions) like used in this study.

The low basal activity of SSAT-EGFP could result from very rapid turnover rate of the protein, like is the case of SSAT itself, or it can be caused by the inefficient translation of the mRNA. In recent studies with the FLAG-tagged SSAT and SSAT fused to *Renilla* luciferase, it was suggested that DENSpm regulates SSAT mRNA translational efficiency by inhibiting a repressor protein from binding to SSAT transcript . In that study, the half-life of the SSAT-luciferase fusion protein in HeLa cells was not affected by DENSpm; only the translation was more effective. Whether DENSpm and CPENSpm- induced accumulation of SSAT-EGFP is affected by the enhanced translation remains to be analyzed. On the other hand, it has been shown earlier that the SSAT protein stability is enhanced by the presence of DENSpm . Studies to reveal the important regions or amino acids of SSAT protein in terms of proteosomal degradation have indicated the importance of carboxyl-terminal glutamic acids at position 170 and 171 (the two last amino acids of SSAT) . Changing these amino acids to either glutamine or lysine or adding two extra lysine residues at the end of the protein rendered the SSAT protein stable. In our study, EGFP was added at the carboxyl-terminus of the protein and thus, could influence the proteosomal degradation of the protein. However, determination of SSAT and SSAT-EGFP half-lives, based on SSAT activity, suggested that in mouse fetal fibroblasts the half-lives of endogenous SSAT and plasmid expressed SSAT-EGFP are very similar and spermine analogs DENSpm and CPENSpm stabilize the both proteins. Interestingly, also CHENSpm seemed to clearly extent the half-live of the fusion protein in the transfected SSAT-deficient mouse fetal fibroblasts, but did not highly affect the half-life of the SSAT protein in the wild-type cells. The reason why CHENSpm do not highly induce the SSAT activity of SSAT-EGFP although it extends the half-life of the protein is not known. One possible explanation can be that the

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configuration of the fusion protein allows CHENSpm to perform stabilization but at the same time the activity of SSAT-EGFP is limited. However, the total protein amounts of SSAT-EGFP and SSAT are not increased by CHENSpm as shown in this study and in addition, the production of variant transcript SSAT-X is increased by the analog in the wild-type cells. In general, it is interesting to realize that EGFP is a relatively stable protein with a half-life of 26 hours. The half-life of SSAT-EGFP being close to the half-life of the wild-type SSAT protein makes SSAT-EGFP an unstable variant of EGFP fusions.

In all, several *N*-alkylated polyamine analogs have been synthesized and evaluated as antitumor agents. Also the analogs used in this study have anti-proliferative or cytotoxic effects. In general, polyamine analogs inhibit cell growth in different ways. One suggested route is the superinduction of SSAT followed by the depletion of intracellular polyamines. As reported previously, the spermine analogs vary in their ability to induce SSAT. In three different human prostate cancer cell lines DENSpm strongly induced SSAT whereas CPENSpm induced it less than DENSpm but clearly more than CHENSpm which gave only a low induction of SSAT activity . Also the intracellular spermidine and spermine levels were less affected by CHENSpm in all three cell lines. In our study, the main purpose was to analyze the regulation of SSAT expression and thus a 24 hours treatment time was used to minimize the growth inhibition or death of the cells by the analogs. However, it needs to be emphasized, that even though cytotoxicity was not detected the reduced EGFP control protein expression in analog-treated cells may reflect decreased cellular functions caused, at least partly, by reduction of intracellular polyamines occurring within this 24 hour treatment time. The reduction of spermidine and spermine pools by all analogs and the slight accumulation of putrescine in CHENSpm and CPENSpm-treated cells as well as the decrease of putrescine level in DENSpm-treated samples were similar in wild-type cells and in the SSAT-EGFP-expressing cells. Interesting results in terms of intracellular polyamine levels in response to the analogs came from the cells expressing EGFP alone. There were no changes in SSAT activity (as expected because the endogenous SSAT is disrupted) but the intracellular polyamine levels were similar to those of wild-type SSAT and SSAT-EGFP expressing cells treated with the analogs. Lower N^1-acetyl-spermidine levels in the EGFP than in the SSAT-EGFP expressing cells showed the absence of SSAT function and, in the case of DENSpm- treated EGFP transfected cells, the lower value of N^1-acetyl-spermidine compared with the untreated and CHENSpm and CPENSpm treated EGFP expressing cells again suggested the strong ability of DENSpm to remove the intracellular polyamines. The loss of intracellular polyamines is interesting since the back-conversion of polyamines needs a functional SSAT protein. Although, spermine oxidase can convert spermine to spermidine it is not able to oxidase spermidine to putrescine . Whether there are still some unknown enzymes in the catabolic pathway of polyamines able to catalyze the back conversion remains to be seen. Earlier it has been shown that DENSpm depletes intracellular polyamines in the mouse embryonic stem cells in which the SSAT gene is disrupted .On the other hand, DENSpm treatment of the SSAT-deficient mouse does not lead to depletion of higher intracellular polyamines in several studied tissues, though the animals are more sensitive to the toxic effects of the analog than their syngenic littermates . In addition of stimulating the catabolic enzymes, the analogs can inhibit polyamine biosynthetic enzymes, thus further lowering the intracellular polyamine pools. In this study the ODC activity was clearly reduced by DENSpm and by CPENSpm in SSAT- deficient cells. However, in wild-type cells only DENSpm decreased ODC activity and, in fact, CPENSpm increased it. We did not study the activity of *S*-adenosylmethionine decarboxylase, AdoMetDC , the other key enzyme in the biosynthesis of polyamines in this study. However, it has been shown earlier that DENSpm and CPENSpm reduce the activity of AdoMetDC, while CHENSpm does not decrease the enzyme activity . Thus, the low intracellular polyamine levels in DENSpm- and CPENSpm-treated cells could partly reflect the reduced biosynthesis but in the case of CHENSpm some additional mechanisms including direct replacement from binding sites and the transport system need to be considered.

In conclusion, the SSAT-EGFP introduced in this paper is encoding a protein with many functional properties similar to SSAT and thus, can be applied to polyamine research. In mouse fetal fibroblasts, the spermine analogs DENspm, CHENSpm and CPENSpm distinctly and systematically regulate the expression of SSAT at multiple levels, including the splicing of pre-mRNA. In addition, these analogs also induce SSAT-independent mechanisms to reduce intracellular polyamines levels. The results from the

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present study may give important information when the functions of SSAT connected to the cytotoxicity are evaluated and for the development and targeting of polyamine structure-based agents for therapeutic purposes.

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FIGURE LEGENDS

Fig. 1. Effects of spermine analogs on the accumulation of SSAT protein in the mouse fetal fibroblasts. A) Mouse SSAT-deficient fetal fibroblasts expressing either EGFP or SSAT-EGFP were grown without or with 10 μM analogs for 24 hours. The proportion of fluorescent protein positive cells was determined with flow cytometry. The expression level was obtained by dividing the mean fluorescence intensity of the EGFP-positive population by the mean fluorescence intensity of the EGFP-negative population. Representative experiment of two is presented. Data are shown as mean ± S.D., where $n = 3$. *$p < 0.05$, **$p < 0.01$ compared with the corresponding untreated control groups. B) The cell lysates of wild-type mouse fetal fibroblasts grown without or with 10 μM analogs for 24 hours were analyzed for the presence of SSAT by western blot. Three independent samples of each treatment are shown. ß-actin was used as a loading control.

Fig. 2. Intracellular localization of EGFP and SSAT-EGFP in analog-treated cells. Control pEGFP-N1 and SSAT-EGFP fusion construct transfected cells were treated with analogs for 24 hours, fixed and counterstained with propidium iodide (PI) to allow nuclei to be visualized. Confocal images were obtained from the same field for detection of EGFP (green) or PI (red) fluorescence. Images were merged to obtain the co-localization of the EGFP and PI (yellow). Note that also the nuclei of untransformed cells are visible.

Fig. 3. Transcription of SSAT-EGFP and EGFP in analog treated cells. (Upper figure) RT-PCR with EGFP derived primers recognizing EGFP and SSAT-EGFP in pEGFP-N1 or SSAT-EGFP construct transfected cells, respectively. The lanes represent independent samples derived from transfected cells grown without or with 10 μM analogs for 24 hours. 18S rRNA was amplified as an internal control. (Lower figure) the intensity of the RT-PCR bands was quantified by densitometry and ROD was determined by dividing the EGFP (representing EGFP or SSAT-EGFP) value by the 18S rRNA value. Data are shown as mean ± S.D., were n = 3. *$p < 0.05$ compared with corresponding untreated control group.

Fig. 4. Accumulation of SSAT and SSAT-X mRNAs in analog treated cells. (Upper figure) SSAT and SSAT-X were amplified by RT-PCR from samples of untreated wild-type mouse fetal fibroblasts or cells treated with 10 μM analogs for 24 hours. 18S rRNA was amplified as an internal control. The lanes represent independent samples. (Lower figure) intensity of the RT-PCR products in the gel was quantified by densitometry. ROD was determined by dividing the SSAT and SSAT-X values by the 18S rRNA value. Data are shown as mean ± S.D. (n = 3). *$p < 0.05$,**$p < 0.01$ compared with untreated control group.

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TABLE 1

Intracellular polyamine pools of EGFP and SSAT-EGFP transfected SSAT-deficient mouse fetal fibroblasts. Cells were grown for 24 hours without or with 10 µM spermine analogs. A representative of two experiments performed in triplicates is shown. Data are expressed as mean ± S.D. (n = 3).

Treatment / Transgene	SSAT activity (pmol/10 min/mg protein)	Polyamine pools (pmol/µg protein)				
		Put	N^1-AcSpd	Spd	Spm	Analog
No analog						
EGFP	39 ± 16	1.5 ± 0.10	0.82 ± 0.05	17.8 ± 0.42	5.6 ± 0.19	NA
SSAT-EGFP	347 ± 112	2.0 ± 0.26	0.88 ± 0.10	15.9 ± 0.16	5.0 ± 0.23	NA
DENSpm						
EGFP	19 ± 19	0.9 ± 0.08[b]	0.65 ± 0.09[a]	5.4 ± 0.38[b]	2.1 ± 0.17[b]	16.3 ± 1.15
SSAT-EGFP	10564 ± 2853[b]	1.1 ± 0.12[b]	0.95 ± 0.11	5.3 ± 0.49[b]	2.4 ± 0.19[b]	17.3 ± 0.98
CHENSpm						
EGFP	59 ± 28	2.4 ± 0.30[b]	0.79 ± 0.05	13.4 ± 0.22[b]	4.4 ± 0.02[b]	10.3 ± 0.92
SSAT-EGFP	1185 ± 197	2.8 ± 0.09[b]	1.44 ± 0.06[a]	12.6 ± 0.66[b]	4.5 ± 0.26[b]	10.4 ± 0.15
CPENSpm						
EGFP	45 ± 1	1.7 ± 0.07	0.79 ± 0.04	10.2 ± 0.42[b]	3.4 ± 0.14[b]	12.8 ± 1.77
SSAT-EGFP	5805 ± 51[b]	2.3 ± 0.06	1.05 ± 0.10	9.6 ± 0.13[b]	3.4 ± 0.07[b]	13.2 ± 1.25

[a] $p < 0.05$, [b] $p < 0.01$, statistical significance of difference between no analog and analog treated cells

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TABLE 2
Intracellular polyamine pools of wild-type mouse fetal fibroblasts. Cells were grown for 24 hours without or with 10 μM spermine analogs. Data are expressed as mean ± S.D. (n = 3).

Treatment	SSAT activity (pmol/10 min/mg protein)	Polyamine pools (pmol/μg protein)				
		Put	N^1-AcSpd	Spd	Spm	Analog
No analog	153 ± 21	2.1 ± 0.12	0.33 ± 0.02	17.5 ± 0.03	5.2 ± 0.12	NA
DENSpm	24579 ± 2598[b]	1.2 ± 0.05[b]	0.55 ± 0.03[b]	2.1 ± 0.06[b]	1.9 ± 0.06[b]	21.8 ± 2.53
CHENSpm	355 ± 56	2.8 ± 0.06[b]	0.63 ± 0.08[b]	15.1 ± 1.20[b]	4.7 ± 0.28[a]	9.2 ± 0.49
CPENSpm	5437 ± 235[b]	3.0 ± 0.10[b]	1.11 ± 0.02[b]	10.4 ± 0.11[b]	3.5 ± 0.04[b]	16.1 ± 1.32

[a] $p < 0.05$, [b] $p < 0.01$, statistical significance of difference between no analog and analog treated cells
NA, not applicable

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TABLE 3
ODC activity in wild-type and SSAT-deficient mouse fetal fibroblasts. Cells were grown for 24 hours without or with 10 μM spermine analogs. Data are expressed as mean ± S.D. (n = 3).

Treatment	ODC activity (pmol/30 min/mg protein)	
	wild-type cells	SSAT-deficient cells
No analog	404 ± 36	393 ± 43
DENSpm	14 ± 1[b]	38 ± 2[b]
CHENSpm	449 ± 16	381 ± 31
CPENSpm	532 ± 49[b]	318 ± 19[a]

[a] $p < 0.05$, [b] $p < 0.01$, statistical significance of difference between no analog and analog treated cells

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TABLE 4
Half-lives of SSAT and SSAT-EGFP detected in the wild-type and in the SSAT-EGFP transfected SSAT-deficient mouse fetal fibroblasts. Mean ± S.D. of three independent experiments performed in triplicates is shown.

	Half-life (min)	
Treatment	SSAT	SSAT-EGFP
No analog	344 ± 81	363 ± 93
DENSpm	1256 ± 287	1287 ± 570
CHENSpm	419 ± 245	1144 ± 276
CPENSpm	668 ± 154	582 ± 98

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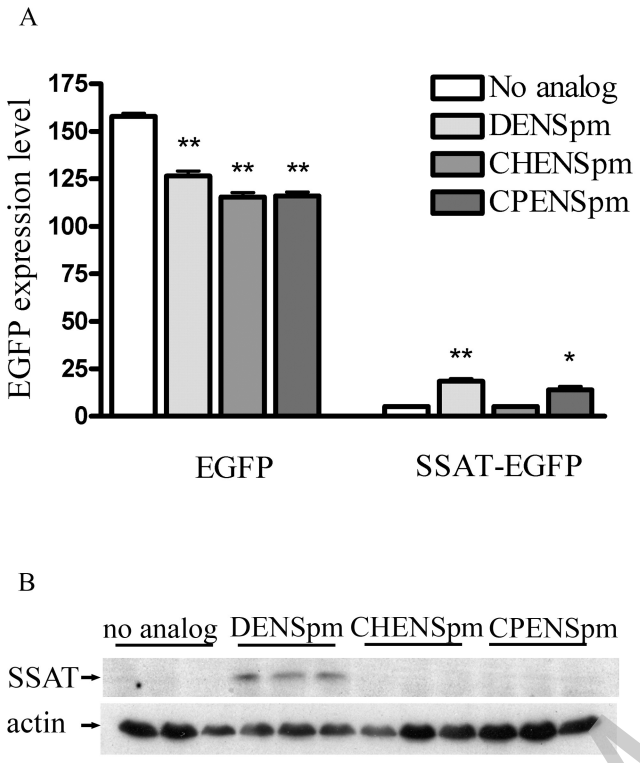

Figure 1.

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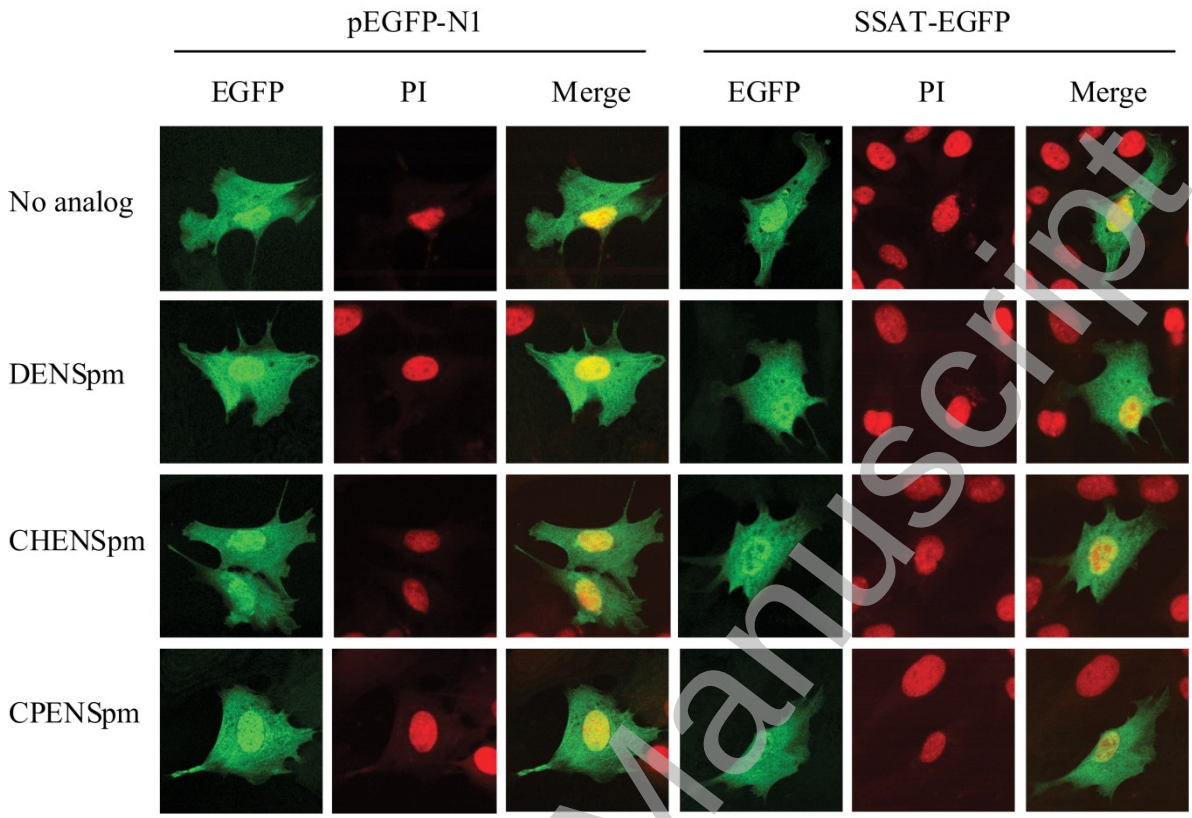

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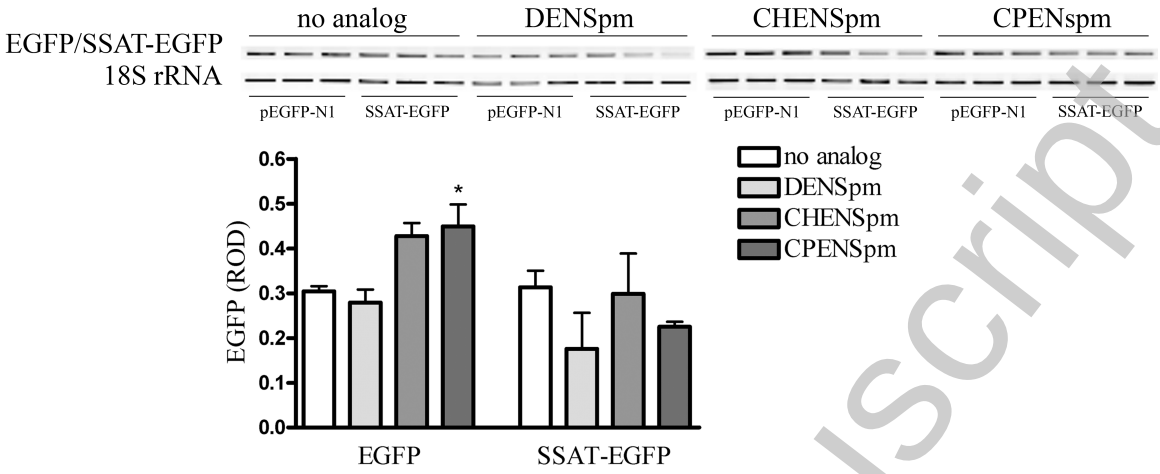

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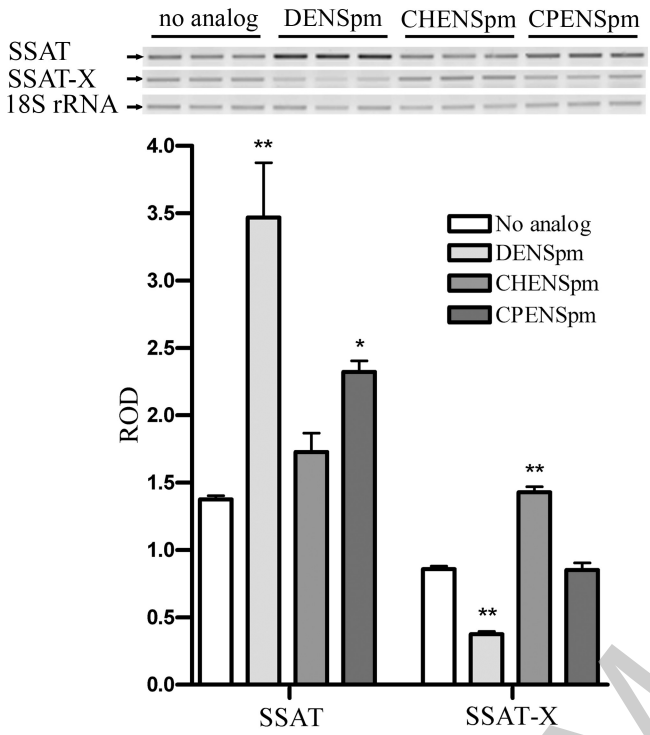

Figure 4.

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