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Small Interfering RNA Suppression of Polyamine Analog-Induced Spermidine/Spermine N^1-Acetyltransferase

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ABSTRACT

N^1,N^{11}-diethylnorspermine (DENSPM) is a polyamine analog that down-regulates polyamine biosynthesis and potently up-regulates the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT). In certain cells, such as SK-MEL-28 human melanoma cells, induction of SSAT is associated with rapid apoptosis. In this study, we used small interfering RNA (siRNA) to examine the role of SSAT induction in mediating polyamine pool depletion and apoptosis. siRNA duplexes were designed to target three independent sites in the SSAT mRNA coding region (siSSAT). When transfected under nontoxic conditions, two of the duplexes selectively reduced basal SSAT mRNA in HEK-293 cells by >80% and prevented DENSPM-induced SSAT mRNA by 95% in SK-MEL-28 cells. Treatment of SK-MEL-28 cells with 10 μM DENSPM in the presence of 83 nM siSSAT selectively prevented the 1400-fold induction of SSAT activity by ~90% and, in turn, prevented analog depletion of spermine (Spm) pools by ~35%. siSSAT also prevented DENSPM-induced cytochrome *c* release and caspase-3 cleavage at 36 h and apoptosis at 48 h as measured by annexin V staining. Overall, the data directly link analog induction of SSAT to Spm pool depletion and to caspase-dependent apoptosis in DENSPM-treated SK-MEL-28 cells. This represents the first use of siRNA technology directed toward a polyamine gene and the first unequivocal demonstration that SSAT induction initiates events leading to polyamine analog-induced apoptosis.

The cellular requirement for polyamines in cell growth is typically met in all cells by a complex system of intracellular polyamine pool maintenance that involves homeostatic balancing of biosynthesis, catabolism, uptake, and export of polyamines. Of these, polyamine catabolism and polyamine export are regulated by an acetylation reaction catalyzed by SSAT. The enzyme acetylates spermine (Spm) and spermidine (Spd) to form N^1-acetylspermine and N^1-acetylspermidine (AcSpd), respectively, which are then either exported out of the cell or degraded to lower polyamines. The latter reaction is catalyzed by polyamine oxidase (PAO), which selectively back-converts N^1-acetyl-SPM to Spd and AcSpd to putrescine (Put) (Seiler, 1987).

The biological potential of these reactions was made obvious during the study of polyamine analogs, such as N^1,N^{11}-diethylnorspermine (DENSPM), that down-regulate polyamine biosynthesis at the level of ornithine decarboxylase (ODC) and potently up-regulate polyamine catabolism at the level of SSAT. The significance of SSAT was clearly implied by the exceptional magnitude of induction observed, which in some cell lines caused increases in enzyme activity of 100- to 1,000-fold. The effect may represent the most potent known gene response to an anticancer agent. The unusual heterogeneity of the response among analog-treated cell lines led to early correlations between growth inhibition and SSAT induction (Porter et al., 1991; Shappell et al., 1992). In addition, correlations were also noted among analogs that differentially induced the enzyme in the same cell line and the extent to which they inhibit cell growth (Kramer et al., 1999). An early attempt to more precisely define this relationship made use of SSAT-targeted antisense in analog-treated cells, an approach that was limited by antisense inefficiency in preventing accumulation of SSAT mRNA and by the fact that a significant portion of SSAT induction occurred post-transcriptionally at the levels of translation and protein stabilization (Fogel-Petrovic et al., 1996b).

At least three genetic approaches have been used to refine this relationship. In the first, murine embryo fibroblasts derived from SSAT transgenic animals were shown to be more growth-inhibited by analog treatment than those from wild-type mice (Alhonen et al., 1998). In the second, McCloskey and Pegg (2000) found that a point mutation in *SSAT* gene was responsible for resistance to DENSPM in a Chinese

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ABBREVIATIONS: Spm, spermine; Spd, spermidine; AcSpd, N^1-acetylspermidine; PAO, polyamine oxidase; Put, putrescine; DENSPM, N^1,N^{11}-diethylnorspermine (also known as DE-333); ODC, ornithine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; DASpm, N^1,N^{12}-diacetylspermine; SMO, spermine oxidase; MDL-72527, N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine; siRNA, small interfering RNA; RNAi, RNA interference; siSSAT, small interfering RNA targeting SSAT; HEK, human embryonic kidney.

hamster ovary cell line. It was subsequently shown that the analog was unable to effectively sustain high levels of SSAT because of its inability to prevent the ubiquitination and rapid turnover of the mutant SSAT protein (McCloskey and Pegg, 2003). Taking an alternative tack, Vujcic et al. (2000) showed that conditional overexpression of SSAT in MCF-7 cells gave rise to a steady depletion of Spd and Spm pools and a gradual inhibition of cell growth. Thus, the linkage between SSAT induction and inhibition of cell growth was firmly established. In addition, that study (Vujcic et al., 2000) identified a new metabolic product, N^1,N^{12}-diacetylspermine (DASpm) that PAO oxidizes to regenerate Put.

Associations between SSAT induction and subsequent apoptotic responses have also been reported (Ha et al., 1997). In characterizing one of the most sensitive human melanoma lines, SK-MEL-28 cells, we found that induction of apoptosis by various polyamine analogs correlates with their ability to induce SSAT (Chen et al., 2001). In both studies, these associations have relied on interference with apoptosis by specific inhibitors of PAO, the enzyme immediately downstream of SSAT in the back-conversion pathway. Such data are built on the fact that PAO liberates hydrogen peroxide and reactive aldehydes such as acetamidopropanol. Thus, it was deduced that up-regulation of the rate-limiting enzyme SSAT may give rise to highly active polyamine catabolism, liberation of the cytotoxic by-products, and inhibition of cell growth because of oxidative damage. Those studies, however, were reported before the discovery of PAO and a new enzyme, spermine oxidase (SMO), which were both found to be analog-inducible, at least at the level of RNA (Wang et al., 2001; Vujcic et al., 2002, 2003;). Because both PAO and SMO give rise to aldehydes and hydrogen peroxide and because both are inhibited by the PAO inhibitor MDL-72527, the earlier apoptosis prevention studies cited above could have been mediated by induced oxidases as opposed to SSAT.

In the absence of specific SSAT inhibitors, the present study makes use of small interfering RNA (siRNA) technology to selectively suppress induction of SSAT activity. RNA interference (RNAi) is the phenomenon of post-transcriptional gene silencing induced by double-stranded RNA that is homologous in sequence to the target gene (Elbashir et al., 2001a,b). In *Drosophila melanogaster*, RNAi involves an initiation step in which longer double-stranded RNA is digested into 21- to 23-nucleotide siRNA duplexes by the enzyme, dicer. In a subsequent effector step, the siRNA duplex binds to its complementary endogenous or heterologous genes and targets this complex for nuclease digestion by an RNA-induced silencing complex (McManus and Sharp, 2002). Artificially designed siRNA duplexes mimic the dicer product and selectively degrade targeted RNA species in mammalian cells via a poorly understood endogenous effector system similar to *D. melanogaster* RNA-induced silencing complex. The most efficient siRNA duplexes are composed of 21-nucleotide sense and antisense strands, paired to have a 19-nucleotide duplex region perfectly matched to the target sequence and containing a two-nucleotide overhang at each 3′ terminus (Elbashir et al., 2001a,b).

In this study, we designed siRNAs to target SSAT as a means to prevent SSAT induction and examine the downstream consequences (or lack thereof) during DENSPM treatment. To our knowledge, this is the first time that this technology applied to a polyamine gene and the first unequivocal demonstration that SSAT induction is essential for initiating events that lead to polyamine analog-induced apoptosis.

Materials and Methods

Materials. The polyamine analog DENSPM was generously provided by Dr. Ron Merriman (Pfizer Pharmaceuticals, Ann Arbor, MI). Polyclonal rabbit anti-caspase-3 and mouse anti-cytochrome *c* antibody were bought from BD Pharmingen (San Diego, CA). SSAT antibody was generated and described previously (Fogel-Petrovic et al., 1996a). Polyamines and acetylated polyamines were purchased from Sigma (St. Louis, MO). DASpm was a gift from Dr. Nikolaus Seiler (Laboratory of Nutritional Oncology, Institut de Recherche Contre les Cancers, Strasbourg, France).

Cell Culture. Human embryo kidney 293 cells (HEK-293) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1 mM aminoguanidine. SK-MEL-28 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% Nu-Serum (Collaborative Research Products, Bedford, MA) and 1 mM aminoguanidine. Cells were maintained in a 5% CO_2 humidified incubator as monolayer cultures.

Transient Transfection. Three siRNA duplexes targeting SSAT (siSSAT-128, −200 and −397) were computer designed and synthesized by Xeragon Inc. (Huntsville, AL). Numbered from the start codon of SSAT mRNA, siSSAT128 targeted nucleotides 128 to 146 (sense, 5′-GAU GGU UUU GGA GAG CAC C-3′; antisense, 5′-GGU GCU CUC CAA AAC CAU C-3′); siSSAT200 targeted nucleotides 200 to 218 (sense, 5′-GGA CAC AGC AUU GUU GGU U-3′; antisense, 5′-AAC CAA CAA UGC UGU GUC C-3′); siSSAT397 targeted nucleotides 397 to 415 (sense, 5′-UGA ACC AUC CAU CAA CUU C-3′; antisense, 5′-GAA GUU GAU GGA UGG UUC A-3′). Scrambled control siRNA that had no sequence homology to any known genes was used as the control. For each 60-mm cell culture dish, 15 μl of the 20 μM stock duplex siSSAT or control siRNA was mixed with 300 μl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 15 μl of LipofectAMINE 2000 (Invitrogen) in 300 μl of Opti-MEM. The solution was incubated for 20 min at room temperature and gently overlaid onto 90% confluent SK-MEL-28 cells in 3 ml of medium. The final concentration of each siSSAT was 83 nM. After 24 h, cells were treated in the absence or presence of 10 μM DENSPM and harvested after another 24 h incubation for polyamine pool, enzyme activity and Northern blot analysis, after 36 h for Western blot analysis, and after 48 h for annexin V staining as a quantitative measure of apoptosis.

Polyamine Pools and Enzymes. Intracellular polyamines and polyamine analogs were analyzed by high-performance liquid chromatography as described previously (Chen et al., 2001). ODC and SSAT activities were assayed as described previously (Porter et al., 1991).

Western Blot Analysis. Cells were harvested and total lysates (40 μg protein/lane) were run on 7.5 to 15% SDS-PAGE gels, followed by transfer to polyvinylidene difluoride membrane and immunoblotted with specific antibodies. Detection was performed with ECL from Amersham Biosciences (Piscataway, NJ). Protein was determined by the standard Bio-Rad assay, and β-actin (Sigma, St. Louis, MO) was used to equalize for variations in loading.

Northern Blot Analysis. Northern blot analysis was carried out as described by Fogel-Petrovic et al. (1993). Briefly, total RNA was extracted with RNeasy Mini Kit (QIAGEN Inc., Valencia, CA). RNA samples (10 μg/lane) were separated on 1.5% agarose/formaldehyde gels and transferred to membrane. The membrane was hybridized to [^{32}P]dCTP-labeled cDNA probes for detection of SSAT mRNA and exposed for autoradiography. The glyceraldehyde-3-phosphate dehydrogenase signal was used as a loading control.

Apoptosis Assay. Apoptosis was analyzed by annexin V/fluorescein isothiocyanate staining using a kit obtained from R&D Systems (Minneapolis, MN). Briefly, cells were harvested and resuspended in

binding buffer at a concentration of 4×10^5 cells/100 μl. For each test, 4×10^5 cells were incubated with propidium iodide (5 μg/ml) and annexin V/fluorescein isothiocyanate (0.25 μg/ml) in the dark for 15 min at room temperature. After incubation, 400 μl of binding buffer was added to stop the reaction, and the tubes were placed on ice. Samples were analyzed within the hour by a BD Biosciences FACScan (Flow Cytometry Facility, Roswell Park Cancer Institute) and data were processed using the Winlist program (Verity House, Topsham, ME).

Determination of Cytosolic Cytochrome *c*. Cells incubated in the presence or absence of 10 μM DENSPM for different periods of time were harvested for separation of mitochondria and cytosol according to the method reported by Yang et al. (1997). Cytochrome *c* was detected on the cytosolic fraction by Western blot analysis.

Results

Selective Decrease of SSAT mRNA by siSSAT. Three siRNA duplexes targeting different locations within the coding region of SSAT mRNA (see *Materials and Methods*) were synthesized and evaluated for their abilities to knock down SSAT expression. Blast search showed that none of the siSSATs shared homology with exons of any other known human genes. A scrambled-nucleotide siRNA was included in each experiment to serve as a control duplex lacking homology with any known gene. We first examined the transfection efficiency in SK-MEL-28 human melanoma cells and found that about 98% cells were transfected (Fig. 1) as described under *Materials and Methods*. Using LipofectAMINE 2000, high cell densities (~90% confluent) were necessary to obtain high transfection efficiency, which limited our ability to assess the influence of SSAT on cell growth kinetics.

We next examined the ability of these siSSAT duplexes to suppress SSAT induction in DENSPM-treated SK-MEL-28 human melanoma cells. This particular line was previously shown to exhibit high SSAT inducibility followed by massive apoptosis when treated with DENSPM (Kramer et al., 1999; Chen et al., 2001). siSSAT128 and siSSAT200 reduced the basal SSAT mRNA levels by 90% relative to control duplex after 48 h transfection (Fig. 2A). After DENSPM treatment for 24 h, SSAT mRNA increased by ~37 fold. Treatment of siSSAT128 or siSSAT200 prevented this increase in mRNA by ~95% (Fig. 2A). By comparison, expression as well as

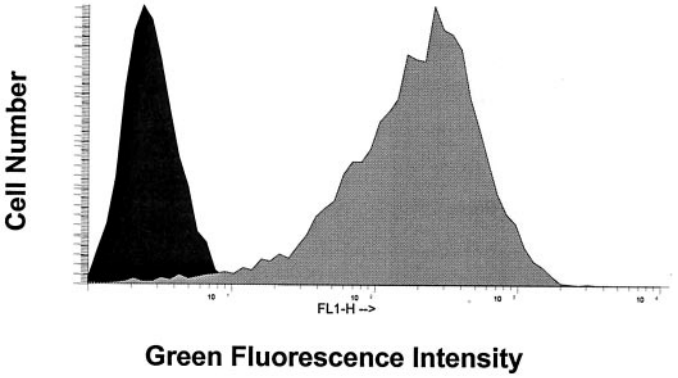

Fig. 1. The transfection efficiency of SK-MEL-28 cells with siRNA. SK-MEL-28 cells were transiently transfected with 83 nM siRNA tagged with green fluorescence protein (GFP) in the presence of LipofectAMINE 2000 for 24 h. Cells were harvested and analyzed by flow cytometry to detect fluorescence intensity (*x*-axis) of 10,000 cells. Black histogram, control cells; gray histogram, siRNA-treated cells.

induction by DENSPM of another polyamine catabolic gene, *SMO* was unaffected.

We also tested these siRNA duplexes targeting SSAT in HEK-293 cells because this particular cell line is known to be highly receptive to transfection, and because it expresses relatively high levels of SSAT mRNA. Control or SSAT-targeted siRNA duplexes at 83 nM were transfected into HEK-293 cells in the presence of 4 μg/ml LipofectAMINE 2000. After 48 h, Northern blot analysis (Fig. 2B) revealed that both siSSAT128 and siSSAT200 reduced SSAT mRNA levels by ~80% relative to control siRNA, whereas siSSAT397 decreased basal levels of SSAT mRNA by ~50%. Because siSSAT200 was slightly better than siSSAT128 in both cell systems, that duplex was chosen for further use.

siSSAT Effects on Polyamine Pools and Enzyme Activities. As shown in Table 1, SSAT activity was reduced by 50% after siSSAT200 treatment, a finding consistent with the fact that SSAT mRNA was reduced to virtually undetectable levels (Fig. 2A). SSAT activity rose from ~20 to ~28,000 pmol/min/mg by DENSPM treatment for 24 h, an increase of ~1400-fold. siSSAT200 prevented this increase in SSAT activity by 90% but had no effect on the polyamine biosynthetic enzyme ODC, which was down-regulated by DENSPM.

There was no significant change in the polyamine pool profile when SK-MEL-28 cells were transfected with either control siRNA or siSSAT200 alone (Table 1). Consistent with previous studies, treatment with 10 μM DENSPM for 24 h decreased Spd and Spm pools by 95% and increased Put, AcSpd, and DASpm pools significantly. These pool changes are known to reflect massive up-regulation of SSAT activity and down-regulation of ODC activity. The relative contribution of SSAT induction to pool changes has not been previously defined. In cells pretreated for 24 h with 83 nM siSSAT200 and then cotreated with the duplex plus 10 μM DENSPM for 24 h, Spm pools were reduced by 60% instead of 95%, whereas Spd pools actually increased, presumably because of DENSPM induction of SMO (Vujcic et al., 2003). The marked accumulation of DASpm and Put seen with DENSPM alone was greatly diminished by siSSAT200. Importantly, we note that DENSPM accumulation was decreased minimally, indicating that the reduction in SSAT activity by siSSAT200 is not caused by reduced DENSPM uptake. In fact, DENSPM levels ranging from ~8,000 to 12,000 pmol/10^6 cells induced similar levels of SSAT in the absence of siRNA (data not shown). In addition, data in Fig. 4B show that siSSAT200 prevents apoptosis at DENSPM concentrations as high as 100 μM.

Requirement of SSAT Induction for Apoptosis. We previously reported that DENSPM caused rapid apoptosis in SK-MEL-28 cells and demonstrated a strong correlation between activation of polyamine catabolism and apoptosis (Chen et al., 2001). Here, we used siSSAT200 to selectively knock-down the SSAT component of polyamine catabolism and examined whether apoptosis is affected at 48 h. As shown in Fig. 3, treatment with control siRNA and DENSPM caused ~50% of the cells to undergo early and late apoptosis by 48 h as indicated by annexin V staining. Control siRNA, siSSAT128, and siSSAT200 alone had no effect on apoptosis in this assay (Fig. 3). Interestingly, when combined with DENSPM, siSSAT128 and siSSAT200 fully prevented apoptosis and maintained cell viability. In Fig. 4, we demonstrate that 83 nM siSSAT200 prevented DENSPM-induced

apoptosis over times up to 48 h of treatment and over a range of DENSPM concentrations up to 100 μM.

We previously showed that DENSPM-induced apoptosis was caspase-dependent (Chen et al., 2001). To further validate the relationship between SSAT induction and apoptosis, we compared the effects of siSSAT200 on DENSPM induction of SSAT protein and apoptotic protein changes. As shown in Fig. 5, induction of SSAT protein, mitochondrial release of

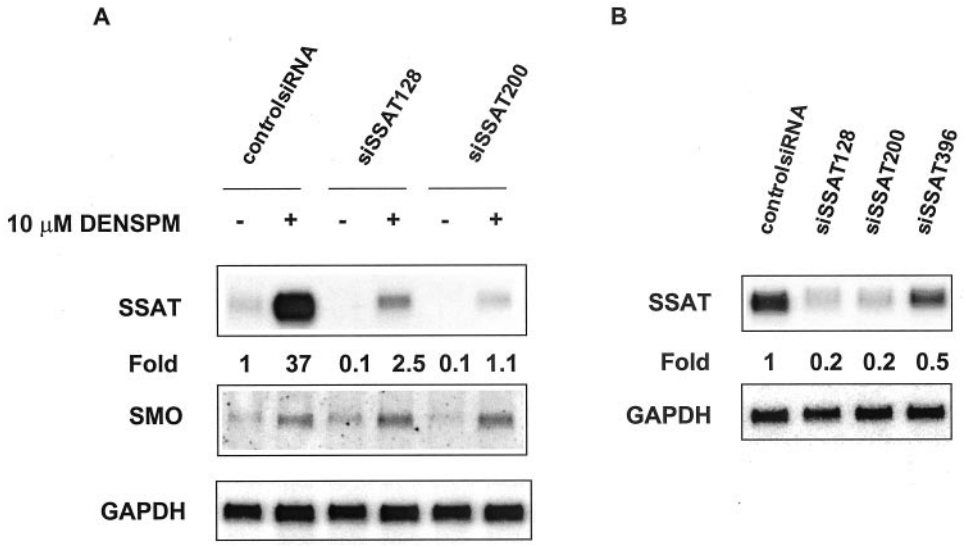

Fig. 2. The effect of siSSAT duplexes on basal and DENSPM-induced SSAT RNA in SK-MEL-28 melanoma cells (A) or on basal SSAT RNA levels in HEK-293 cells (B). A, SK-MEL-28 cells were transfected and then treated with 10 μM DENSPM for the last 24 h before Northern analysis at 48 h. B, HEK-293 cells were transfected with either control siRNA or three different siSSAT at 83 nM in the presence of 4 μg/ml LipofectAMINE 2000 and harvested for Northern blot analysis after 48 h. Data are representative of two separate experiments. SMO and glyceraldehyde-3-phosphate dehydrogenase were included to serve as control genes.

TABLE 1
Effects of SSAT-directed siRNA on DENSPM-induced changes in polyamine metabolism in SK-MEL-28 cells

Treatment	Enzyme Activity		Polyamine Pools					
	SSAT	ODC	Put	AcSpd	Spd	DASpm	Spm	DENSPM
	pmol/min/mg	*nmol/h/mg*	*pmol/10^6 cells*					
Control siRNA	20 ± 2	0.47 ± 0.12	<10	<10	650 ± 360	<10	5,290 ± 320	
siSSAT200	12 ± 5	0.54 ± 0.04	<10	<10	870 ± 265	<10	5,250 ± 210	
Control siRNA + DENSPM	28,040 ± 3,380	0.05 ± 0.01	685 ± 225	465 ± 40	30 ± 15	135 ± 45	325 ± 80	11,766 ± 2908
SiSSAT200 + DENSPM	2,910 ± 550	0.03 ± 0.01	230 ± 130	330 ± 30	1,385 ± 70	<10	2,020 ± 195	7308 ± 766

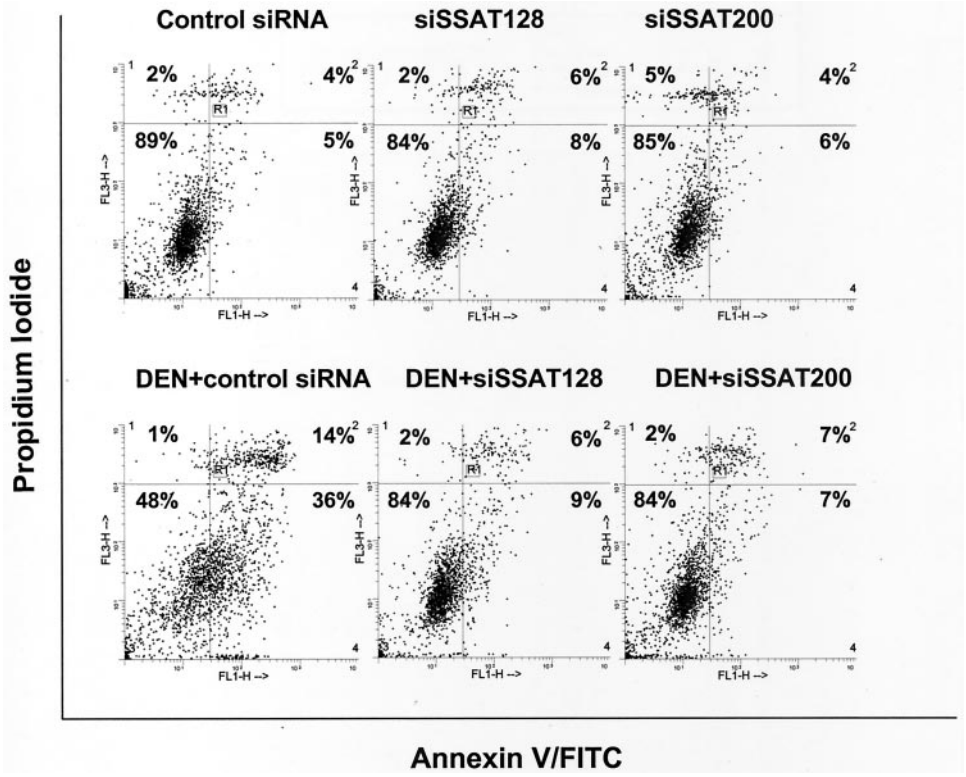

Fig. 3. Prevention of DENSPM-induced apoptosis in SK-MEL-28 cells by siSSAT. SK-MEL-28 cells were transfected with either 83 nM control siRNA, siSSAT128, or siSSAT200. Twenty-four hours after transfection, cells were treated with 10 μM DENSPM (DEN) for another 48 h. Cells were then harvested and subjected to annexin V staining and flow cytometric analysis. Data are representative of at least two separate experiments run in duplicate.

cytochrome *c*, and caspase-3 activation by DENSPM were effectively blocked by siSSAT200. This indicates that both effectors in the apoptotic cascade are dependent on upstream events initiated by SSAT, and that siSSAT200 was able to protect cells against DENSPM challenge.

Discussion

RNA interference is the process whereby double-stranded RNA inhibits gene expression in a sequence-dependent fashion. In the natural state, RNAi is thought to contribute to antiviral defense and to the post-transcriptional regulation of gene expression (McManus and Sharp, 2002). However, it has repeatedly been shown that introduction of artificial siRNA duplexes can effectively and selectively silence specific genes in mammalian cells (McManus and Sharp, 2002), suggesting that exogenous siRNA may find usefulness in gene-specific therapeutics and/or in defining gene function.

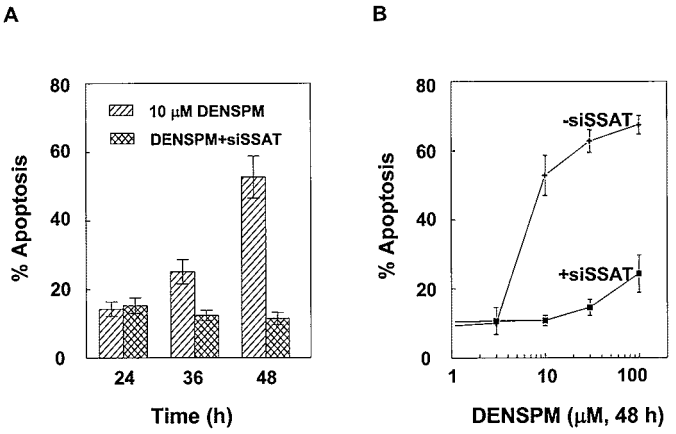

Fig. 4. siRNA prevention of time- (A) and dose- (B) dependent induction of apoptosis by DENSPM in SK-MEL-28 cells. SK-MEL-28 cells were transfected with either control siRNA or siSSAT200 at 83 nM. Twenty-four hours after transfection, cells were treated with 10 μM DENSPM for the various times (A) or with various concentrations of DENSPM for 48 h (B). Cells were then harvested and subjected to annexin V staining and flow cytometric analysis. Data are representative of three separate experiments.

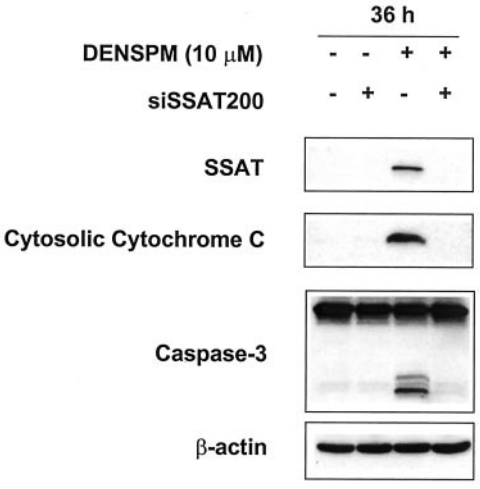

Fig. 5. Prevention of DENSPM-induced cytochrome *c* release and caspase-3 activation by siSSAT200. SK-MEL-28 cells were transfected with either control siRNA or siSSAT200 at 83 nM. Twenty-four hours after transfection, cells were treated with 10 μM DENSPM for another 36 h. Cells were then harvested and subjected to Western blot analysis using the antibodies that detect SSAT, cytochrome *c* or caspase-3. Data are representative of two separate experiments. β-actin served as a loading control.

The present findings indicate that SSAT is quite vulnerable to RNAi approaches. Basal levels of SSAT mRNA were reduced in HEK-293 and SK-MEL-28 cells, whereas those of SMO, another polyamine gene induced by DENSPM, were not affected. Of the three coding regions selected, the siRNA targeting nucleotides 200 to 218 was most effective possibly because this middle region is less likely to be bound with regulatory proteins. As an aside, there was no indication during the 2-day incubation that the suppression of basal SSAT levels was, by itself, growth inhibitory. This finding is consistent with the fact that SSAT-deficient mouse embryonic stem cells proliferate normally (Niiranen et al., 2002).

Importantly, the siRNA duplexes effectively suppressed the potent 37-fold induction of SSAT mRNA by DENSPM. The findings suggest that targeted RNA degradation is sufficient to overcome substantial increases in SSAT gene transcription and mRNA stabilization by DENSPM (Fogel-Petrovic et al., 1993). The effects also extended to the level of SSAT activity. SSAT-directed siRNA prevented DENSPM-induced SSAT activity (~1400-fold) by 90%. A significant portion of the remaining SSAT is expected to be bound and inhibited by DENSPM (Pegg et al., 1990). In the absence of any known specific inhibitors of SSAT, the RNAi approach provides a viable means for exploring the metabolic function of this protein and its relationship to cell growth.

Comparison of polyamine pools from cells treated with DENSPM plus targeted or control siRNAs provided new insights into the contribution of SSAT to polyamine pool depletion during DENSPM treatment. First, it is important to note that SK-MEL-28 cells are somewhat unusual in their basal Spm to Spd pool ratios. Whereas most cells typically contain similar amounts of Spm and Spd, SK-MEL-28 cells have >8-fold more Spm than Spd. Thus, siSSAT200 prevented DENSPM depletion of both Spm and Spd pools. In fact, the loss of Spm was prevented by ~35% and Spd actually increased (~2-fold) above control levels. Put pools were also increased above control levels. These latter two findings most probably reflect the SMO- and PAO-mediated back-conversion of Spm to Spd and Spd to Put, respectively. In cells treated with DENSPM plus siSSAT200, the absence of the metabolite DASpm further indicates a loss in functional enzyme activity. Unexpectedly, DENSPM pools in cells treated with DENSPM plus siSSAT200 were ~30% less than those treated with DENSPM plus control siRNA. We speculate that this modest decrease in accumulation reflects the occupation of DENSPM intracellular binding sites by the natural polyamines and, thus, reduced analog accumulation within the cell.

We have shown previously that SK-MEL-28 cells undergo apoptosis in response to DENSPM treatment and that this involves an initial release of cytochrome *c* from mitochondria followed by activation of caspase-9 and caspase-3 as part of the intrinsic apoptotic cascade (Chen et al., 2001). More recently, we have shown that this is preceded by a down-regulation of the antiapoptotic protein survivin and melanoma inhibitor of apoptosis protein (Chen et al., 2003). In both cases, the relationship of these findings to SSAT was supported by the observation that analogs that differentially induce SSAT bring about apoptosis in a manner that corresponds directly with their ability to induce enzyme. Although

these data strongly imply a role for SSAT in apoptosis, they must now be qualified by the recent observations that these same analogs also differentially induce PAO and SMO mRNA and, presumably, enzyme activity (Wang et al., 2001, 2002; Vujcic et al., 2003). This recently discovered caveat formed the rationale for the present studies. Thus, we demonstrate that selective interference of DENSPM-induced SSAT prevents apoptotic signaling and apoptosis indicated by activation of caspase-3 and annexin V staining, respectively. Although the findings do not exclude the possibility that this is ultimately related to oxidative disturbances caused by by-products of the PAO reaction, they clearly show that the events are initiated by induction of SSAT and not by SMO or PAO. Likewise, the findings also indicate that down-regulation of ODC and *S*-adenosylmethionine decarboxylase is not the initiating event in apoptosis, although they may be considered contributory. In a separate publication, we have further demonstrated that siSSAT200 was able to prevent DENSPM-induced IAP degradation (Chen et al., 2003). These findings are unambiguous and unique in the SSAT literature.

It is interesting that the siRNAs are effective at very low doses (83 nM) relative to those of DENSPM (10 μM). In a dose-response experiment, we observed that siRNA was able to protect cells against DENSPM treatment for 48 h up to 100 μM. Importantly, we found that the siRNA is most effective if cells are pretreated for 24 h with the duplexes before exposure to DENSPM. Presumably, this is because of reductions in basal levels of SSAT mRNA that are available for translation. Even with comparable manipulations and higher concentrations, we were not previously able to achieve a similarly effective response with SSAT-directed antisense molecules (Fogel-Petrovic et al., 1996b).

In summary, the polyamine data are supportive of a scenario whereby SSAT induction is suppressed by siRNA leading to a reduced depletion of Spm and Spd pools during the first 24 h. Apoptosis would seem to be dependent on the extent to which Spm is lost in SK-MEL-28 cells, and the data further suggest that critical Spm levels lies between 100 and 40% of control. Although SSAT is the initiating event, some downstream activity must give rise to growth inhibition. Possibilities include a rapid loss of functional polyamines and/or the generation of hydrogen peroxide and aldehydes, by-products of the SSAT-coupled PAO caused by the increased availability of acetylated polyamine substrates. This latter possibility is supported by studies in which DENSPM treatment is combined with the PAO inhibitor MDL-72527, giving rise to reduced apoptosis (Chen et al., 2001; Ha et al., 1997).

Taken together, these data suggest that induction of SSAT by polyamine analogs is critical for initiating downstream apoptotic events. Many polyamine analogs have been described previously (Thomas et al., 2002) and several have been (or are being) advanced to clinical trials as anticancer agents (Creaven et al., 1997; Streiff and Bender, 2001; Hahm et al., 2002; Frydman et al., 2003). Most tend to be pleiotropic in their mode of drug action, and multiple mechanisms of growth inhibition have been put forth. However, on the basis of the data presented here, DENSPM is the first polyamine analog to be assigned a singular dominating drug effect (i.e., SSAT induction) that is unequivocally linked to a specific type of growth inhibition (apoptosis). Thus, in at least certain cell types, DENSPM behaves as a targeted anticancer agent. With the recent realization that many anticancer drugs such as platinum agents are potent nonspecific inducers of SSAT expression (Hector et al., 2002), it may be possible to enhance therapeutic efficacy via drug combinations involving cytotoxics and selective inducers of SSAT.

Acknowledgments

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