

The role of spermidine/spermine N^1 -acetyltransferase in determining response to chemotherapeutic agents in colorectal cancer cells

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Abstract

Polyamines have been shown to play a role in the growth and survival of several solid tumors, including colorectal cancer. We identified the polyamine catabolic enzyme *spermidine/spermine N¹-acetyltransferase (SSAT)* as being one of the most highly inducible genes in two DNA microarray screens to identify novel determinants of response to chemotherapeutic agents in colorectal cancer. SSAT was shown to be inducible in response to 5-fluorouracil (5-FU) or oxaliplatin in parental and drug-resistant HCT116 cell lines. It was also shown that SSAT mRNA was up-regulated in response to 5-FU or oxaliplatin in a panel of six colorectal cancer cell lines. The polyamine analogue N^1,N^{11} -diethylnorspermine (DENSpm) depletes polyamine pools and potently induces SSAT. We evaluated the effect of combining DENSpm with chemotherapeutic agents in HCT116 p53^{+/+} cells and in HCT116 drug-resistant daughter cell lines. Western blot analyses showed that SSAT protein expression was dramatically enhanced when DENSpm was combined with oxaliplatin or 5-FU in HCT116 p53^{+/+} cells. Using cell viability assays and flow cytometry, synergistic induction of cell death was observed following cotreatment of HCT116 p53^{+/+} cells with DENSpm and each

chemotherapeutic agent. Of note, this combined therapy increased the chemosensitivity of cells rendered resistant to each of these chemotherapeutic agents. Small interfering RNA-mediated down-regulation of SSAT resulted in loss of synergy between DENSpm and these agents. These results show that SSAT plays an important role in regulating cell death following combined cytotoxic drug and DENSpm treatment. Furthermore, DENSpm sensitizes both sensitive and resistant cells to chemotherapeutic agents. Taken together, these results suggest that SSAT may be an important target for therapeutic intervention in colorectal cancer. [Mol Cancer Ther 2007; 6(1):128–37]

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in the western world. Approximately 25% of colorectal cancer patients present with metastatic disease, and between 40% and 50% of newly diagnosed patients eventually develop metastatic disease.

The antimetabolite 5-fluorouracil (5-FU) is the most extensively used chemotherapy against this malignancy. However, in the metastatic setting, only 10% to 15% of patients derive benefit from 5-FU monotherapy (1). Combination of 5-FU with the topoisomerase I inhibitor irinotecan (CPT-11) or the DNA-damaging agent oxaliplatin has significantly improved response rates to 40% to 50% in the advanced setting (2, 3). More recently, novel agents, such as the monoclonal antibodies cetuximab, panitumumab, and bevacizumab, which target the epidermal growth factor receptor and vascular endothelial growth factor receptor, respectively, have become available and have shown promising activity in metastatic colorectal cancer (4–6). However, the prognosis for many patients still remains poor, and this is frequently due to chemoresistance, which is either intrinsic or acquired during treatment.

DNA microarray technology is a valuable tool to investigate the molecular basis of drug resistance at the level of gene expression. Previous studies in our laboratory have employed this technique to identify 5-FU-inducible target genes in MCF-7 breast cancer cells (7) and to compare differential gene expression in HCT116 p53^{+/+} colorectal carcinoma cells and 5-FU- and oxaliplatin-resistant sublines (8). Interestingly, the polyamine catabolic enzyme *spermidine/spermine N¹-acetyl transferase (SSAT; EC 2.3.1.57)* was identified as being one of the most highly drug inducible genes in both studies and therefore warranted further investigation.

SSAT is the rate-limiting enzyme in polyamine catabolism. The polyamines putrescine, spermidine, and spermine are essential for cell growth and differentiation, and

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their levels are highly regulated. The key enzymes in the biosynthetic pathway are S-adenosyl methionine decarboxylase (SAM-DC; EC 4.1.1.50) and ornithine decarboxylase (ODC; EC 4.1.1.17). Spermidine and spermine synthase catalyze the transfer of aminopropyl groups in the conversion of putrescine to spermidine and spermidine to spermine, respectively. SSAT is involved in the transfer of acetyl groups from acetyl CoA to form the N^1 -acetyl-derivatives of spermine or spermidine. These are then oxidized by polyamine oxidase (PAO; EC 1.5.3.11) to produce spermidine and putrescine, respectively. Byproducts of the oxidation steps are H_2O_2 and 3-acetamidopropanol, both of which have been shown to be cytotoxic (9, 10). N^1 -acetyl spermidine can also be exported from the cell, which contributes to polyamine pool depletion. The more recently discovered catabolic enzyme spermine oxidase (SMO, PAOh1) has the ability to directly oxidize spermine, converting it back to spermidine (11, 12). Under normal conditions, polyamines regulate their own biosynthesis and prevent overproduction. In cancer cells, polyamine concentrations are elevated (13–16), as are acetylpolyamines (17), suggesting a link between polyamine regulation and carcinogenesis. The polyamine pathway is therefore an attractive target in the development of anticancer strategies.

A number of inhibitors, targeting individual enzymes in the polyamine pathway, exist (for reviews, see refs. 18, 19). The major weakness of these compounds has been their inability to deplete all three polyamines sufficiently to decrease cell survival. Polyamine analogues, such as the symmetrically substituted analogue N^1,N^{11} -diethylnorspermine (DENSpm), are structural derivatives of natural polyamines. This allows their uptake by the polyamine transporter; however, they are not functional substitutes. They have the capability to deplete all three intracellular polyamines as they down-regulate ODC and SAM-DC by negative feedback, decrease polyamine uptake, and potentially induce SSAT. In some cases, the polyamine analogues may induce SMO, the activity of which has also been implicated in drug response (11, 20). This leads to increased polyamine catabolism and export. This “superinduction” of SSAT is the result of increased transcription, translation, and stabilization of the protein that normally has a short half-life of ~30 min (21).

Early-phase clinical studies carried out in patients with previously treated metastatic breast cancer showed that although DENSpm was quite well tolerated, there was no evidence of clinical activity (22, 23). This disappointing outcome could be due to a number of factors, including the administration schedule, patient selection, or the type of malignancy treated. Encouraging preclinical data for polyamine analogues alone and in combination with cytotoxic drugs support their continued evaluation. The efficacy of combining polyamine analogues with cytotoxics has been evaluated in a number of *in vitro* studies (24–26). It has been postulated that treatment with a cytotoxic may transcriptionally induce SSAT, and that further treatment with a polyamine analogue relieves

translational repression of SSAT to induce active SSAT protein (25).

The aim of the current study was to evaluate the role of SSAT, which was recently identified in a DNA microarray screen carried out in our laboratory (27) in regulating the response to the combination of DENSpm and chemotherapeutic agents in colorectal cancer cell line models.

Materials and Methods

The polyamine analogue DENSpm was purchased from Tocris (Ellisville, MO). A 10 mmol/L stock solution was prepared in sterile injection water and stored at -20°C . Further dilutions were made using sterile PBS. 5-FU and oxaliplatin were obtained from Sigma Chemical Co. (St. Louis, MO) and Sanofi-Synthelabo (Malvern, PA), respectively. 5-FU was prepared in sterile PBS, and oxaliplatin was prepared using sterile injection water. Both were stored as 1 mmol/L stock solutions at 4°C . Further dilutions were made using sterile PBS.

Tissue Culture

The p53 wild-type HCT116 human colon cancer cell line was kindly provided by Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). The 5-FU- and oxaliplatin-resistant HCT116 sublines and the p53 mutant R248W and R175H HCT116 cell lines were generated in our laboratory as previously described (8, 28). All HCT116-derived cell lines were maintained in McCoy's 5A medium supplemented with 10% dialyzed FCS, 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. RKO, H630, and HT-29 cells (obtained from the National Cancer Institute, Bethesda, MD) were maintained in DMEM and supplemented as above. LoVo cells (kindly provided by AstraZeneca, Macclesfield, United Kingdom) were maintained in DMEM supplemented as above minus sodium pyruvate (all medium and supplements from Invitrogen Life Technologies Corp., Paisley, Scotland). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2 .

Cytotoxicity/Combination Index Assays

Cells in the exponential growth phase were seeded at 2,000 per well in 96-well microtiter plates in drug-free media. Twenty-four hours later, cells were treated with a range of concentrations of 5-FU or oxaliplatin in the presence or absence of 0.1, 1, and 10 $\mu\text{mol}/\text{L}$ DENSpm. Cells were also treated with a range of DENSpm alone. After 72 h, 25 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (5 mg/mL) from Sigma Chemical were added to each well. The plates were incubated at 37°C for 2 h before the culture medium was removed, and formazan crystals were reabsorbed in 200 μL of DMSO. Cell viability was determined by reading the absorbance of each well at 570 nm using a Biotrak II Plate reader (Amersham Biosciences, Bucks, United Kingdom).

Flow Cytometry

Cells were seeded at 1×10^5 per well in six-well tissue culture plates. After 24 h, cells were treated with a range of concentrations of 5-FU or oxaliplatin \pm 1 $\mu\text{mol}/\text{L}$ DENSpm

for 72 h. DNA content of harvested cells was evaluated after propidium iodide staining of cells using an EPICS XL Flow Cytometer (Coulter, Miami, FL).

Western Blot Analyses

Western blots were done as described previously (29) using a rabbit polyclonal antibody raised against purified recombinant human SSAT (30) in conjunction with a horseradish peroxidase-conjugated sheep anti-rabbit secondary antibody (Amersham Little Chalfont, Buckinghamshire, United Kingdom). Equal loading was assessed using a β -tubulin mouse monoclonal primary antibody (Sigma-Aldrich, St. Louis, MO).

Real-time Reverse Transcription-PCR Analysis

Total RNA was isolated using the RNA STAT-60 reagent (Biogenesis, Poole, United Kingdom) according to the manufacturer's instructions. Reverse transcription was carried out with 1 μ g of RNA using an MMLV-based reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR amplification was carried out in a final volume of 10 μ L containing 5 μ L of 2 \times Sybr Green Master mix, 4 μ L of primers (2 μ mol/L), and 1 μ L of cDNA using a DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA). mRNA levels for SSAT and the internal standard (18S) were measured in triplicate wells and in triplicate assays. SSAT mRNA was detected using Quantitect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), and 18S mRNA was detected using DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). Reaction conditions were activation at 95°C for 15 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and stop reaction at 75°C for 1 s. PCR amplifications were done for 40 cycles, and melt curve analysis was used to examine the specificity of amplified product. Standard curves were generated to quantify the absolute expression levels of the SSAT gene and the 18S rRNA reference gene in each sample. The relative expression level of each gene in samples of interest was calculated by dividing the amount of normalized target by the value in an untreated calibrator sample.

The primers for SSAT and 18S were purchased from Invitrogen Life Technologies: SSAT forward primer, GCT-AATTCGTGATCCGC; SSAT reverse primer, CAATGCT-GTGTCTTCCG; 18S forward primer, CATTCGTATT-GCGCCGCTA; 18S reverse primer, CGACGGTATCTGAT-CGTCT.

All cell lines were seeded at 1×10^6 per 90-mm tissue culture dish and treated with their respective $IC_{50(72\text{ h})}$ doses of DENSp_m, oxaliplatin, or 5-FU, alone or in combination with 1 μ mol/L DENSp_m, for 24, 48, and 72 h.

Small Interfering RNA Transfection

The small interfering RNA (siRNA) duplex targeting SSAT nucleotides 200 to 218 (sense, 5'-GGACACAGCAUUGUUGGUU-3'; antisense, 5'-AACCAACAAUGCUGUGUCC-3') was chosen as previously described (31) and obtained from Dharmacon, Inc. (Lafayette, CO). The control siRNA (SC) target sequence used was AATTCTCCGAACGTGTCACGT.

Cells were seeded onto 96-well plates at 5,000 per well for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, or at 1×10^6 in 90-mm tissue culture dishes for protein extraction in McCoy's 5A media plus 10% dFCS. siRNA transfections were done on subconfluent cells incubated in unsupplemented Opti-MEM using the oligofectamine reagent (both from Invitrogen) according to the manufacturer's instructions. Cells were drug-treated 4 h after transfection and analyzed by Western blot and cell viability analyses.

DNA Microarray and Data Analysis

HCT116 parental cells were treated with 5 μ mol/L 5-FU or 1 μ mol/L oxaliplatin for 0, 6, 12, and 24 h. Untreated 5-FU- and oxaliplatin-resistant cells were also analyzed to allow identification of constitutively dysregulated genes relative to the untreated (0 h) parental line.³ Microarray data analysis was done using GeneSpring v7.1 (Agilent Technologies UK Ltd., Stockport, United Kingdom) as previously described (27).

Statistical Analysis

Correlation coefficients (R^2) were calculated using Pearson correlation coefficient. The statistical significance of R^2 was calculated using a two-tailed test of significance (SPSS 11.0 for Windows). Synergy was assessed using Calcsyn software (Biosoft, Cambridge, United Kingdom) to determine combination index (CI) values, which are a measure of drug interaction effect. All drug treatments were done in triplicate wells, and all assays were done in triplicate. Synergy was defined as any CI <1; additivity was defined as CI = 1; and antagonism was defined as CI >1. Two-way ANOVA calculations were also carried out using a 2 \times 2 factorial design (SPSS11.0 for Windows).

Results

Effects of Cytotoxic Drug Treatment on SSAT mRNA Expression

We have previously generated a panel of drug-resistant HCT116 cell lines (8) and used them in a DNA microarray experiment to identify novel determinants of chemoresistance (27). Microarray data were analyzed and validated by quantitative PCR to assess the basal and drug-inducible expression of SSAT mRNA in HCT116 p53^{+/+} parental, oxaliplatin- and 5-FU-resistant colorectal cancer cell lines. Quantitative PCR data showed strong correlations with microarray data, with SSAT induced by ~7-fold in the HCT116 parental cells ($R^2 = 0.85$) and ~2.5-fold in the 5-FU-resistant subline ($R^2 = 0.98$) following treatment with 5 μ mol/L 5-FU (Fig. 1A). Following treatment with 1 μ mol/L oxaliplatin, quantitative PCR data showed that SSAT mRNA was induced by ~18.5-fold in the HCT116 parental cells and more moderately induced by ~3-fold in the oxaliplatin-resistant subline following treatment with 1 μ mol/L oxaliplatin (Fig. 1B). Again, this correlated well

³ Detailed experimental protocols and raw expression data are available at <http://www.ebi.ac.uk/arrayexpress/> (accession no. E-MEXP-390).

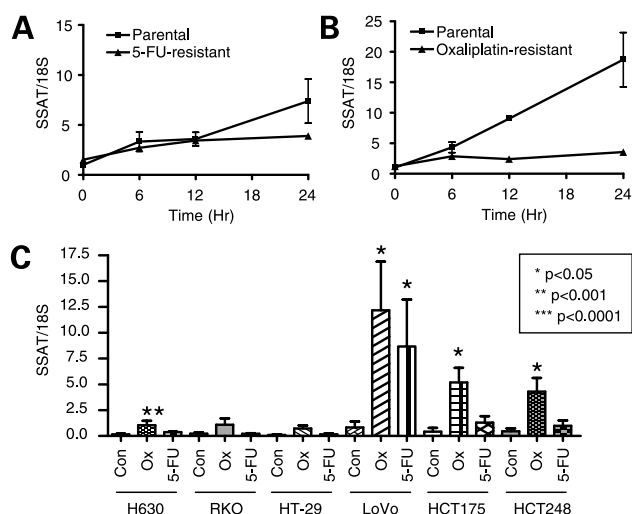


Figure 1. SSAT mRNA expression measured by quantitative reverse transcription-PCR. **A**, HCT116 p53^{+/+} parental and 5-FU-resistant sublines were treated with 5 μmol/L 5-FU for 6, 12, and 24 h. **B**, HCT116 p53^{+/+} parental and oxaliplatin-resistant cells were treated with 1 μmol/L oxaliplatin for 6, 12, and 24 h. **C**, H630, RKO, HT-29, LoVo, HCT175, and HCT248 colorectal cancer cells were treated with IC_{50(72h)} doses of oxaliplatin or 5-FU for 24 h. Each experiment was done in triplicate, and data were normalized to 18S.

with the microarray data ($R^2 = 0.996$ and $R^2 = 0.72$, respectively).

In addition to these studies, the cytotoxic drug-induced levels of SSAT mRNA were also examined in a panel of six other colorectal cancer cell lines (Fig. 1C). These cell lines were selected due to the differing status of the p53 gene. The panel chosen consisted of H630 (p53 mutation on exon 10), RKO (p53 wild type), HCT175 (p53 mutation at codon 175), HCT248 (p53 mutation at codon 248), HT-29 (p53 mutation at codon 273), and LoVo (p53 wild type) colorectal cancer cells. The cell lines expressed the similar constitutive levels of SSAT mRNA (Fig. 1C). SSAT mRNA was inducible in all six colorectal cancer cell lines in response to IC_{50(72 h)} doses of oxaliplatin at 24 h, with the greatest induction occurring in the LoVo cells followed by the HCT175 and HCT248 cells. It was observed that IC_{50(72 h)} doses of 5-FU produced little induction of SSAT mRNA in the H630, RKO, and HT-29 cells. A greater induction of SSAT mRNA was noted in the HCT175, HCT248, and the LoVo cells; however, this was not as great as the induction seen following oxaliplatin treatment (Fig. 1C).

Effects of Combining the Polyamine Analogue DENSpm with Cytotoxic Drug on Cell Viability

The polyamine analogue DENSpm is a structural derivative of natural polyamines, which has the ability to deplete polyamine pools and has been evaluated in clinical trials (22, 23). We analyzed the effects of combining DENSpm and cytotoxic drug on the viability of HCT116 colorectal cancer cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assays were carried out, and the data analyzed according to Chou and Talalay (32) to obtain CIs, which are a measure of drug

interaction effect. Scheduling experiments were carried out to determine the optimum conditions and adding both agents simultaneously resulted in the greatest effect (data not shown). HCT116 p53^{+/+} cells were treated simultaneously with either 0.1, 1, or 10 μmol/L DENSpm in combination with either 0.5, 5, or 10 μmol/L oxaliplatin or 0.5, 5, or 10 μmol/L 5-FU for 72 h. Strong synergistic interactions were observed at all three DENSpm concentrations with all oxaliplatin concentrations used (CI range, 0.24–0.74), except for the 0.1 μmol/L DENSpm/10 μmol/L oxaliplatin combination (CI = 1.27; Fig. 2A, I). Strongly synergistic interactions were also observed when 0.1, 1, or 10 μmol/L DENSpm was combined with any of the 5-FU concentrations used (CI range, 0.24–0.73), the one exception being the 10 μmol/L DENSpm/0.5 μmol/L 5-FU combination (CI = 1.13; Fig. 2A, II). These studies were also completed in HCT116 p53^{-/-} cells. The combination of oxaliplatin with DENSpm produced highly synergistic interactions in all combinations (CI range, 0.07–0.99; Fig. 2A, III). 5-FU and DENSpm cotreatment also resulted in strongly synergistic interactions, producing CIs < 1 (range, 0.08–0.66) for all combinations used with the exception of the 10 μmol/L DENSpm/0.5 μmol/L 5-FU treatment (Fig. 2A, IV).

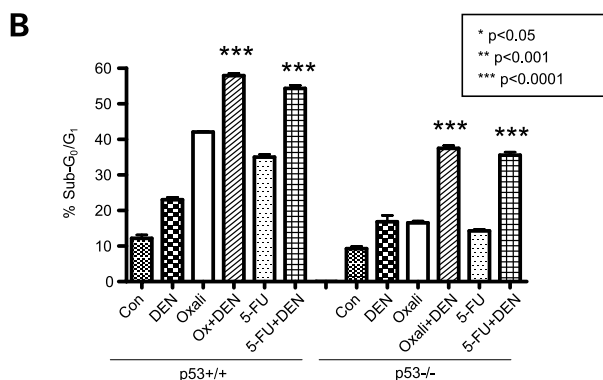
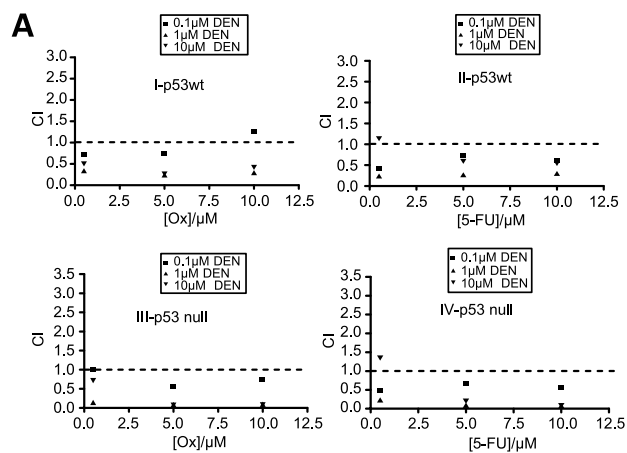
Flow cytometric analysis showed that combined treatment of either the p53^{+/+} or p53^{-/-} HCT116 cells with 1 μmol/L DENSpm and either 1 μmol/L oxaliplatin or 5 μmol/L 5-FU resulted in a greater than additive increase in the sub-G₀-G₁ apoptotic peak compared with either drug alone (Fig. 2B; ref. 27). This suggests that the synergistic interaction between chemotherapeutic agents and DENSpm in these cell lines is due to enhanced apoptosis. Furthermore, it shows that p53 status alone does not play a role in regulating the interaction between chemotherapeutic agents and DENSpm.

In addition to the HCT116 p53^{+/+} and p53^{-/-} cell lines, cell viability analyses were done in a further six colorectal cancer cell lines: the H630, LoVo, HCT248, HCT175, RKO, and HT-29 cell lines (Fig. 2C). The results showed that the combination of oxaliplatin and DENSpm produced a highly synergistic effect in the H630, HCT175, and HCT248 cells and a synergistic to highly synergistic effect in the RKO cells. Although the combination of oxaliplatin and DENSpm produced a synergistic effect in the HT-29 cells, an additive to synergistic interaction was observed in the LoVo cells. The combination of 5-FU and DENSpm produced a highly synergistic effect in the H630, HCT175, and HCT248 cells and a synergistic effect in the LoVo, RKO, and HT29 cells.

Effects of Combining DENSpm and Chemotherapeutic Agents on SSAT mRNA and Protein Expression

HCT116 p53^{+/+} cells were treated with 1 μmol/L DENSpm, 1 μmol/L oxaliplatin, or 5 μmol/L 5-FU alone, or with combinations of 1 μmol/L DENSpm for 24, 48, and 72 h. These concentrations represent IC_{50(72 h)} values for each drug in this cell line. Using quantitative PCR, analysis showed a statistically significant increase in SSAT mRNA expression following treatment with the combination of

oxaliplatin and DENSpm at all three time points ($P < 0.001$ at 24 h, $P < 0.0001$ at 48 h, $P < 0.001$ at 72 h; Fig. 3A). It was shown that treatment with the combination of DENSpm and 5-FU produced a statistically significant increase in



C

	Oxali/DENSpm	5-FU/DENSpm
H630	+++	+++
LoVo	+ / ++	++
HCT248	+++	+++
HCT175	+++	+++
RKO	++ / +++	++
HT-29	++	++

Figure 2. **A**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assays in (I) HCT116 p53^{+/+} colorectal cancer cells cotreated for 72 h with DENSpm and oxaliplatin, (II) HCT116 p53^{+/+} colorectal cancer cells cotreated for 72 h with DENSpm and 5-FU, (III) HCT116 p53^{-/-} colorectal cancer cells cotreated for 72 h with DENSpm and oxaliplatin, (IV) HCT116 p53^{-/-} colorectal cancer cells cotreated for 72 h with DENSpm and 5-FU. Results are expressed as CIs, where CIs < 1 indicate synergistic effects. **B**, flow cytometric analysis showing the percentage of cells in the sub-G₀-G₁ phase of the cell cycle in the HCT116 p53^{+/+} and p53^{-/-} colorectal cancer cells following combined treatment of DENSpm and either 1 μmol/L oxaliplatin or 5 μmol/L 5-FU. **C**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assays were used to determine CIs for the interaction between DENSpm with oxaliplatin or 5-FU in H630, LoVo, HCT248, HCT175, RKO, and HT-29 colorectal cancer cells. + + +, highly synergistic; + +, synergistic; +, additive; -, antagonistic. Representative of three separate experiments.

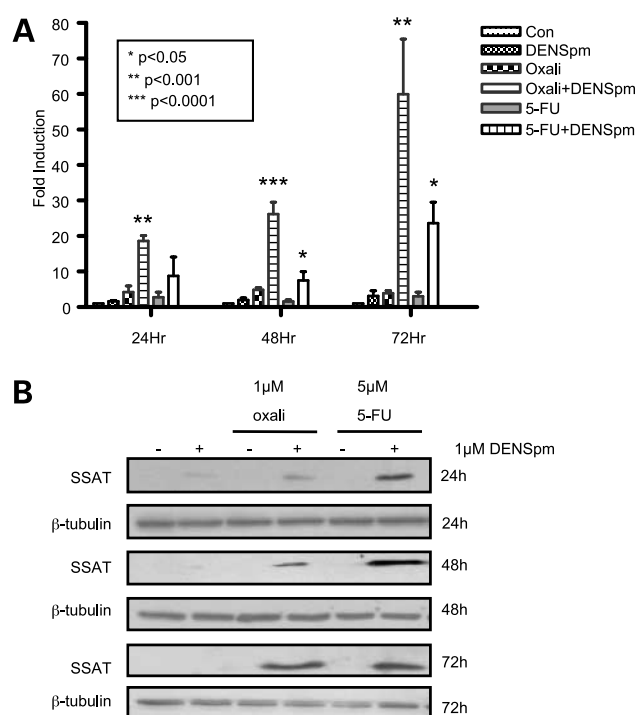


Figure 3. SSAT mRNA expression measured by quantitative reverse transcription-PCR (**A**) and Western blot analysis of SSAT protein expression (**B**) in HCT116 p53^{+/+} parental cells following treatment with 1 μmol/L DENSpm, 1 μmol/L oxaliplatin, or 5 μmol/L 5-FU, alone or with combinations of 1 μmol/L DENSpm with each chemotherapeutic agent for 24, 48, and 72 h. Each experiment was done in triplicate.

SSAT mRNA at 48 and 72 h, with the greatest effect observed at 72 h ($P < 0.05$ at 48 h, $P < 0.05$ at 72 h; Fig. 3A). Furthermore, Western blot analysis revealed that there was a greater-than-additive induction of SSAT protein expression when oxaliplatin or 5-FU were combined with DENSpm at any time point compared with either treatment alone (Fig. 3B), with the highest levels of induction occurring at 72 h for oxaliplatin/DENSpm and 48 h for 5-FU/DENSpm.

Effects of SSAT siRNA on the Synergistic Interaction between DENSpm and Chemotherapeutic Agents

To examine the role that SSAT plays in modulating the synergistic interaction between chemotherapeutic agents and DENSpm in HCT116 parental cells, the effect of SSAT-targeted siRNA on the combination of DENSpm and either 5-FU or oxaliplatin was examined. As shown in Fig. 4A, treatment with 1 nmol/L SSAT siRNA knocked down SSAT protein following cytotoxic drug and DENSpm cotreatment. Furthermore, the synergistic interaction observed between oxaliplatin and DENSpm was lost when the *SSAT* gene was silenced, with CI increased from 0.55 (synergistic) to 1.30 (antagonism; Fig. 4B). Similarly, a loss in synergistic interaction between 5-FU and DENSpm was also shown following knock down of SSAT, with CI increased from 0.58 (synergistic) to 0.99 (additive; Fig. 4B). These data show the requirement for SSAT induction in the

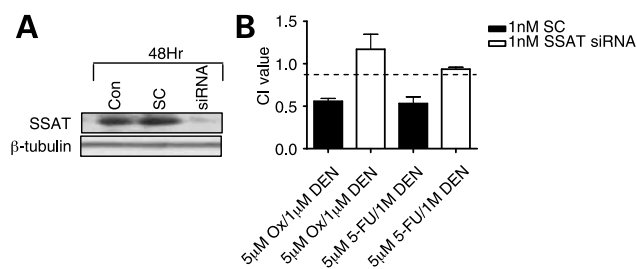


Figure 4. **A**, Western blot analysis showing knock down of SSAT protein in the presence of 1 $\mu\text{mol/L}$ DENSpm using 1 nmol/L siRNA at 48 h in HCT116 p53^{+/+} colorectal cancer cells. **B**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assays were used to measure CIs for interactions between 5 $\mu\text{mol/L}$ oxaliplatin or 5 $\mu\text{mol/L}$ 5-FU with 1 $\mu\text{mol/L}$ DENSpm, in the presence and absence of SSAT siRNA in the HCT116 p53^{+/+} colorectal cancer cells. Each experiment was done in triplicate.

synergistic response following combined treatment with DENSpm and cytotoxic drug.

Effects of Combining DENSpm and Chemotherapeutic Agents on HCT116 Drug-Resistant Cell Survival

We were interested to determine if we could use this combination therapy approach to resensitize our previously generated drug-resistant HCT116 cell lines (8). Treatment of the HCT116 p53^{+/+} oxaliplatin-resistant cells with the combination of DENSpm and either 10 or 20 $\mu\text{mol/L}$ of oxaliplatin gave a greater-than-additive induction of SSAT protein compared with either treatment alone (Fig. 5A). HCT116 p53^{+/+} 5-FU-resistant cells treated with the combination of either 5 or 10 $\mu\text{mol/L}$ 5-FU and 1 $\mu\text{mol/L}$ DENSpm also gave a greater-than-additive induction of SSAT protein compared with either treatment alone (Fig. 5A).

Cell viability was assessed in the both the HCT116 p53^{+/+} oxaliplatin- and 5-FU-resistant cells following treatment with 0.1, 1, or 10 $\mu\text{mol/L}$ DENSpm in combination with chemotherapeutic agents for 72 h. The lowest CIs, indicative of strong synergy, were observed when 1 $\mu\text{mol/L}$ DENSpm was combined with any concentration of oxaliplatin or 5-FU used (CI range, 0.33–0.45; Fig. 5B).

Flow cytometric analysis showed enhanced apoptosis, as shown by an increase in the sub-G₀-G₁ peak, in the HCT116 p53^{+/+} oxaliplatin- and 5-FU-resistant cell lines when 1 $\mu\text{mol/L}$ DENSpm was combined with 5 $\mu\text{mol/L}$ oxaliplatin or 10 $\mu\text{mol/L}$ 5-FU, respectively, in comparison with each therapy alone (Fig. 5C). These results indicate that DENSpm enhances cytotoxic drug-induced apoptosis, even in drug-resistant cell lines.

Discussion

Recently, a DNA microarray screen was carried out in our laboratory to identify genes that may regulate resistance to 5-FU and oxaliplatin in colorectal cancer cells (27). Bioinformatic analyses showed that one of the most highly inducible genes in the HCT116 parental cells by both 5-FU and oxaliplatin was the polyamine catabolic enzyme SSAT.

Furthermore, SSAT was also found to be inducible following drug treatment in both the 5-FU- and oxaliplatin-resistant HCT116 sublines. Real-time PCR validation of SSAT expression showed that there was a very strong correlation between the microarray results and the quantitative PCR results in both the parental and resistant lines following drug treatment. These findings were of particular interest, as an earlier cDNA microarray experiment done in our laboratory to investigate changes in the transcriptional profile of MCF-7 breast cancer cells in response to 5-FU, also identified SSAT as being highly inducible (~13-fold) following treatment with 10 $\mu\text{mol/L}$ 5-FU. This study also showed that SSAT was highly inducible in response to oxaliplatin and the antifolate Tomudex in MCF-7 cells (7).

Real-time PCR revealed that SSAT mRNA expression was up-regulated by 5-FU and oxaliplatin treatment in the HCT116 parental cells. In their respective resistant settings, SSAT was also induced by 5-FU and oxaliplatin treatment. In addition, we examined the inducibility of SSAT in a panel of colorectal cancer cell lines following treatment with either 5-FU or oxaliplatin. The results revealed that both 5-FU and oxaliplatin induced SSAT mRNA expression in the H630, HT-29, LoVo, HCT175, and HCT248 colorectal cancer cell lines; however, only oxaliplatin induced SSAT in RKO colorectal cancer cells. Furthermore, it was noted that oxaliplatin was a much more potent inducer of SSAT mRNA compared with 5-FU in this panel of cell lines.

Both 5-FU and oxaliplatin have been previously shown to up-regulate p53 (29); however, there did not seem to be an association between p53 and SSAT induction in response to cytotoxic drug. The LoVo cells produced the greatest up-regulation of SSAT mRNA following either 5-FU or oxaliplatin treatment. However, the RKO cells, which are also p53 wild type, exhibited the lowest induction of SSAT in response to either drug. Furthermore, the HCT175 and HCT248 cells, which are both p53 mutant, retained the ability to significantly up-regulate SSAT mRNA following treatment with either oxaliplatin or 5-FU. However, studies previously done in our laboratory observed that inactivation of p53 in an MCF-7-derived cell line resulted in significantly reduced levels of 5-FU-mediated induction of SSAT (7). This study also showed that SSAT contains three putative p53-binding sites, which suggested the probability of p53 playing a role in the regulation of SSAT expression. Choi et al. did not observe a significant difference in the induction of SSAT mRNA between HCT116 p53 wild type and p53 null cells in response to 5-FU (26). Taken together, these results would suggest that there does not seem to be an association between p53 status and SSAT regulation in response to 5-FU or oxaliplatin in colorectal cancer cell line models.

In addition to previous studies carried out in our laboratory that showed that SSAT mRNA was inducible in a panel of cell lines in response to 5-FU, oxaliplatin, and Tomudex (7), other studies have shown that both 5-FU and doxorubicin also induce SSAT activity (34). Hector et al. carried out a study to investigate changes in the transcriptional profile of A2780 ovarian carcinoma cells exposed to

cisplatin or oxaliplatin. They observed that SSAT was one of the top 10 genes induced by oxaliplatin and among the top 20 induced by cisplatin (25). In addition, Babbar et al. found that SSAT was induced by 4-fold following sulindac sulfone treatment in CaCo-2 cells (35). The observations that SSAT is highly inducible in response to chemotherapeutic agents and other agents encouraged us to investigate the potential role that SSAT may play in drug resistance/response.

The polyamine pathway is tightly regulated by ODC, SAM-DC, and SSAT, as disruption of the polyamine pool leads to cell cycle arrest and apoptosis (36, 37); therefore, targeting polyamine levels has been studied as a therapeutic approach in cancer. DENSp_m acts by producing a down-regulation of the polyamine biosynthetic enzymes ODC and SAM-DC and also potently up-regulating SSAT. This results in intracellular polyamine pool depletion and eventually cell death. Bernacki et al. generated preclinical data for DENSp_m in a number of human solid tumor xenografts (38). They observed that DENSp_m had similar activity in all but one of the cell lines studied (IC₅₀, 0.1–

1 μmol/L), the exception being the HT-29 cells that had an IC₅₀ >100 μmol/L for DENSp_m. In our studies, we noted that the HT-29 cells had a relatively low basal level of SSAT mRNA, which may explain this finding. Single-agent polyamine analogues have been shown to have antitumor activity in a range of tumor types, including breast, lung, bladder, pancreatic, leukemia, and melanoma (24, 39–43).

To investigate the functional significance of polyamine metabolism in modulating the response to chemotherapeutic agents in colorectal cancer, we combined DENSp_m with 5-FU or oxaliplatin. Scheduling experiments were carried out to determine the optimum conditions for combining chemotherapeutic agents and DENSp_m. Schedules included (a) adding cytotoxic drug and DENSp_m simultaneously for 72 h, (b) adding cytotoxic drug 24 h before addition of DENSp_m for a further 48 h, and (c) adding DENSp_m 24 h before addition of cytotoxic drug for a further 48 h. Strong synergistic interactions were obtained using all three schedules. Therefore, adding DENSp_m and cytotoxic drug simultaneously was used for all combination therapy experiments in this study. Furthermore, other groups have

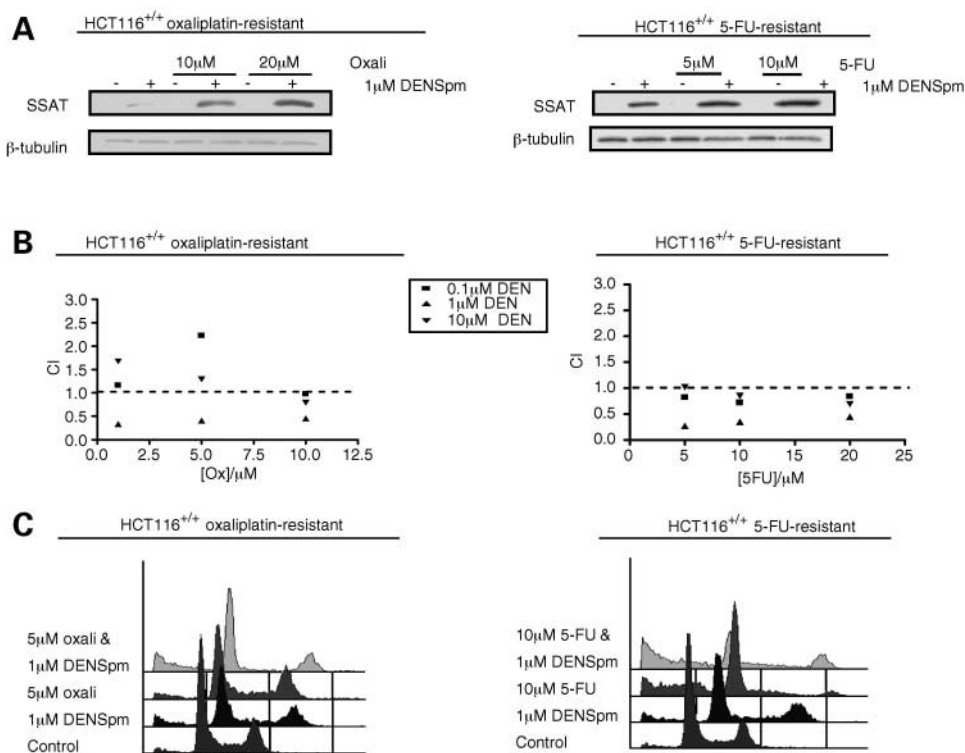


Figure 5. **A**, Western blot analysis of SSAT protein expression in HCT116 p53^{+/+} oxaliplatin-resistant cells following treatment with 1 μmol/L DENSp_m, 10 μmol/L oxaliplatin, or 20 μmol/L oxaliplatin, alone or with combinations of 1 μmol/L DENSp_m with either concentration of oxaliplatin for 72 h and in HCT116 p53^{+/+} 5-FU-resistant cells following treatment with 1 μmol/L DENSp_m, 5 μmol/L 5-FU, or 10 μmol/L 5-FU, alone or with combinations of 1 μmol/L DENSp_m with either concentration of 5-FU for 72 h. β-Tubulin was measured as a loading control. **B**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assays in HCT116 p53^{+/+} oxaliplatin-resistant cells cotreated with DENSp_m and oxaliplatin for 72 h and in HCT116 p53^{+/+} 5-FU-resistant cells cotreated with DENSp_m and 5-FU for 72 h. Results are expressed as CIs, where CIs < 1 indicate synergistic effects. Representative of three separate experiments. **C**, flow cytometric analysis showed enhanced apoptosis when DENSp_m was combined with oxaliplatin in the HCT116 p53^{+/+} oxaliplatin-resistant cells for 72 h and 5-FU in the HCT116 p53^{+/+} 5-FU-resistant cells for 72 h. Representative of three separate experiments.

investigated the effect of scheduling using DENSp_m with either 5-FU (26) or oxaliplatin (25). Choi et al. assessed several treatment schedules in the HCT116 cell line model and observed that the optimum effect resulted from the simultaneous addition of the agents and noted that the most moderate effect resulted from adding DENSp_m before 5-FU (26). Hector et al. assessed varying treatment schedules in A2780 ovarian cancer cells and observed the greatest effect following simultaneous treatment with oxaliplatin and DENSp_m followed by DENSp_m for an additional 24 h.

In the HCT116 p53^{+/+} cell line, we observed a synergistic induction of SSAT mRNA following treatment with 5-FU or oxaliplatin combined with DENSp_m compared with either treatment alone. In the p53^{+/+} cells, the induction of SSAT mRNA was also accompanied by a superinduction of SSAT protein expression and a strong synergistic decrease in cell viability following the combined treatment of cytotoxic drugs and DENSp_m. Cell cycle analyses showed that treatment with oxaliplatin or 5-FU in combination resulted in enhanced cell death compared with either treatment alone in both the p53^{+/+} and p53^{-/-} cells. To ensure that this synergistic interaction was not a cell type-specific event, we also examined the effects of combining cytotoxic drugs and DENSp_m in a wider panel of colorectal cancer cell lines with differing p53 status. We found that the combination of each chemotherapeutic agent and DENSp_m resulted in synergistic interactions in all of the six cell lines tested; therefore, this effect did not seem to be a cell type- or p53-specific phenomenon.

Other studies have considered the efficacy of combining polyamine analogues, such as DENSp_m, with standard cytotoxic agents. Hector et al. showed that oxaliplatin treatment in A2780 ovarian cancer cells potently up-regulated SSAT, and that this response, together with growth inhibition, was synergistically enhanced by cotreatment with DENSp_m (25). Choi et al. observed a synergistic increase in SSAT mRNA levels and cell death in response to 5-FU and DENSp_m combination therapy in HCT116 p53^{+/+} and p53^{-/-} colorectal cancer cells (26). Studies by Hahm et al. showed several synergistic combinations between several different polyamine analogues and several chemotherapeutic agents in a panel of breast cancer cell lines (24). The efficacy of using DENSp_m as an anticancer therapy has been investigated in stage I and II clinical trials. Phase I trials were carried out in 29 patients with advanced non-small cell lung cancer (22). The investigators concluded that DENSp_m could be safely administered with minimal toxicity; however, no disease response was observed. Clinical studies have also assessed the tumor specificity of DENSp_m monotherapy in lung and breast cancer. The results showed that the induction of SSAT by DENSp_m monotherapy was specific to the tumor as they only detected minimal staining for SSAT in the surrounding normal tissue (44, 45). Phase II clinical trials were undertaken in 16 metastatic breast cancer patients previously treated with first-line chemotherapy and/or trastuzumab (23). In this trial, the dose and administration schedule of DENSp_m was reasonably well tolerated;

however, no clinical activity was observed. It was hypothesized that this lack of response might be due to patient selection or administration schedule and suggested that the favorable safety profile of the polyamine analogue class of compounds might make them suitable candidates for combination with standard cytotoxic agents. Indeed, the encouraging results from our *in vitro* studies that have assessed cotreating cell lines with DENSp_m and cytotoxic drugs warrant further investigation first in *in vivo* studies, and if the results are still encouraging and the combinations well tolerated, then they could be taken forward into clinical trials in colorectal cancer. The issues that need to be addressed in future *in vivo* studies are as follows: (a) What is the best schedule to give these two agents? (b) Are these compounds well tolerated when given as a combined therapy? (c) Is this combined therapy tumor specific, and what is the effect on normal tissues? (d) Is there a synergistic response to these combined agents? If the goals of these *in vivo* studies are realized, and if statistically significant results are obtained, the synergistic interaction between DENSp_m and chemotherapy could be translated into an effective treatment strategy in patients.

In this study, we were able to knock down SSAT levels following DENSp_m treatment using a SSAT-specific siRNA. Interestingly, these studies showed that knocking down SSAT to basal levels resulted in loss of synergy between DENSp_m and either oxaliplatin or 5-FU. These data confirm the role of SSAT induction in synergistically enhancing cell death in response to the combined treatment of DENSp_m with these chemotherapies. These studies also suggest that the apoptotic effect is not due to either DENSp_m induction of the oxidases (PAO or SMO) or DENSp_m down-regulation of the decarboxylases (ODC or SAM-DC) and therefore suggests that SSAT plays an important role in regulating cell death following combined chemotherapeutic agent and DENSp_m treatment.

Most interestingly, we also investigated the synergistic interaction in HCT116 p53^{+/+} 5-FU-resistant and oxaliplatin-resistant daughter cell lines. The results of these studies showed that the combination of chemotherapeutic agent and DENSp_m resulted in a greater-than-additive induction of SSAT protein in all cell lines compared with either treatment alone. In addition, these combinations resulted in a synergistic decrease in cell viability; however, this was not evident at all concentrations. Lastly, flow cytometric analyses showed that the combination of chemotherapeutic agent and DENSp_m sensitized the drug-resistant cell lines to cytotoxic drug-induced cell death as measured by the increase in sub-G₀-G₁ apoptotic fraction. Interestingly, Marverti et al. observed a synergistic interaction following combined treatment with BESp_m and cisplatin (CDDP) in CDDP-sensitive and CDDP-resistant ovarian carcinoma cells (46). This study found that the CDDP-resistant cells were also cross-resistant to BESp_m; however, when they were evaluated following concurrent treatment of both agents, they observed a synergistic decrease in cell viability, and the cells became resensitized to cytotoxic drug-induced cell death.

In conclusion, SSAT has become an important target for anticancer strategies for a number of reasons. First, the polyamines play a fundamental role in the cell growth and differentiation in both normal and tumor cells. Second, ODC and SAM-DC are overexpressed in tumor versus normal cells, therefore potentially leading to a survival advantage in tumor cells due to increased capacity to synthesize polyamines (47, 48). Third, the high induction of SSAT following treatment with polyamine analogues is a tumor-specific event (44, 45). In terms of our study, one of the most important facets is that it has shown that treatment of drug-resistant cell lines with a combination of chemotherapeutic agent and polyamine analogues reversed the drug-resistant phenotype. Therefore, DENSPm is able to sensitize both sensitive and resistant colorectal cancer cells to chemotherapeutic agents (46). p53 has been reported to be mutated in 40% to 60% of colorectal cancers and has been described as the universal sensor of genotoxic stress (49). p53 status has been studied as a prognostic factor and, more recently, as a predictor of response to chemotherapy; therefore, we assessed if p53 played a role in response to the combination of DENSPm and chemotherapeutic agents. By using a panel of colorectal cancer cell lines with differing p53 status, we were able to show that the combination of DENSPm and chemotherapeutic agents produced synergistic interactions in all cell lines tested, therefore suggesting that the interaction is a p53-independent event, a highly important finding in terms of colorectal cancer. Finally, our SSAT-specific siRNA studies have clearly shown that SSAT is the key target involved in the synergistic interaction between chemotherapeutic agents and DENSPm. Taken together, these data strongly suggest that combining DENSPm and chemotherapeutic agents may be a useful anticancer therapy; however, these studies will need to be tested further, in *in vivo* models, to examine if the synergistic interaction between DENSPm and chemotherapeutic agents still exist.

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