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ORIGINAL ARTICLE

Polyamine catabolism in colorectal cancer cells following treatment with oxaliplatin, 5-fluorouracil and N^1, N^{11} diethylnorspermine

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Abstract
Purpose Our previous studies showed that combined treatment of oxaliplatin and N^1, N^{11}diethyl-norspermine (DENSPM) results in massive induction of spermidine/spermine N^1-acetyltransferase (SSAT) mRNA and activity. Since oxaliplatin and 5-fluorouracil (5FU) are used clinically in treatment of colorectal cancers, this study examines the effect of adding DENSPM to oxaliplatin/5FU combination on SSAT and spermine oxidase (SMO) in HCT-116 cells.
Methods HCT-116 cells were treated with clinically relevant concentrations of drugs for 20 h followed by 24 h in drug free medium. SSAT and SMO mRNA and protein were assayed by QRT-PCR and Westerns respectively; polyamine pools were measured by HPLC. SSAT and SMO mRNA in tumor biopsies from patients with rectal cancer receiving oxaliplatin, capecitabine and radiation were measured by QRT-PCR.
Results Oxaliplatin + 5FU + DENSPM produced significantly higher levels of SSAT and SMO mRNA, protein and activity than those seen with oxaliplatin+5FU with a significant depletion of cellular spermine and spermidine pools. Oxaliplatin/DENSPM was superior to 5FU/DENSPM in SSAT induction but similar for SMO. Oxaliplatin + DENSPM revealed synergistic growth inhibition at $>IC_{50}$ concentrations and antagonism at $<IC_{50}$. SMO and SSAT induction occurred in 60 and 30% of the patient samples examined.
Conclusions These studies demonstrated that combining DENSPM with oxaliplatin + 5FU provides an added benefit by aiming at the clinically relevant therapeutic target, the polyamine catabolism. Further, we show for the first time, that SMO and SSAT induction could be measured in tumor biopsies in patients receiving chemo-radiation. Optimization of treatment conditions *in vivo* should facilitate a clinical evaluation of the three drug combination.

Keywords Oxaliplatin · 5Fluorouracil · Diethylnorspermine · Polyamine catabolism · Colon cancer cells

Abbreviations

DENSPM	N^1N^{11}-Diethylnorspermine
ERCC-1	Excision cross-complementing gene-1
5FU	5-Fluorouracil
dUMP	Deoxyuridine monophosphate
Pt	Platinum
Put	Putrescine
QRT-PCR	Quantitative RT-PCR
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N^1-acetyltransferase (also known as SSAT-1)
SMO	Spermine oxidase
SRB	Sulforhodamine-B
TMP	Thymidine monophosphosphate
TP	Thymidine phosphorylase

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TS	Thymidylate synthase
FdUMP	5-Fluorouridine monophosphate

Introduction

The platinum (Pt) drugs oxaliplatin and cisplatin are important in the treatment of various types of cancer. Cisplatin, the prototype Pt drug, is active against a variety of tumor types, including tumors of the testes, ovaries, head and neck and bladder. Oxaliplatin, a third generation Pt agent is active in the treatment of colon cancer, a tumor type known to be non-responsive to cisplatin based therapies [40]. Colon cancer is traditionally treated with the anti-metabolite 5FU. However, since the response rates are low for the single agent, 5-fluorouracil (5FU) is used in combination with oxaliplatin as first line treatment of stages II and III colorectal cancers as a means to improve response rates.

In vitro cytotoxicity studies of the NCI drug-screening panel demonstrate that oxaliplatin and cisplatin have different sensitivity profiles, indicating different mechanisms of action and/or resistance [33]. Both oxaliplatin and cisplatin are believed to exert their cytotoxic action via the formation of Pt-DNA adducts which interfere with the replication and transcription processes of the cell, leading to cell death. While these Pt-DNA adducts are believed to be the major contributor to Pt drug cytotoxicity, these agents interact with other molecules in the cell such as RNA and protein, and the implications of these interactions are not fully understood. Our recent gene expression profiling studies and further validations with real time QRT-PCR showed that Pt drugs, especially oxaliplatin, cause an up-regulation of the polyamine acetylation enzyme spermidine/spermine N^1 acetyltransferase (SSAT) expression in A2780 ovarian carcinoma cells [20, 21, 37].

Polyamines are small cationic molecules that are essential for cell proliferation and survival. SSAT is the key enzyme in polyamine catabolism and induction of this enzyme leads to polyamine pool depletion, inhibition of cell growth and eventual apoptosis [2, 8]. Intracellular polyamine levels are known to be elevated in tumor cells compared to normal cells [32], making their metabolism a potential therapeutic target. Strategies to down-regulate polyamine biosynthesis by drugs such as difluoromethylornithine have been tested clinically but were unsuccessful [1]. The polyamine analog N^1, N^{11} diethylnorspermine (DENSPM) targets the polyamine pathway by up-regulating catabolism via SSAT induction and simultaneously decreasing their biosynthesis by down-regulating the biosynthetic enzymes ornithine decarboxylase and *S*-adenosylmethionine decarboxylase [35]. DENSPM has been tested in several Phase I clinical trials [12, 18, 34] and a Phase II study [41] and is currently undergoing further clinical evaluation.

Studies in our laboratory revealed that potent induction of SSAT by oxaliplatin occurs mainly at the level of mRNA and does not always result in a proportional increase in enzyme activity. However, when combined with DENSPM, a synergistic up-regulation of SSAT mRNA and a massive increase in SSAT activity occurs resulting in a near-total depletion of cellular polyamine pools [21]. In addition to SSAT, oxaliplatin up-regulates both oxidative enzymes polyamine oxidase (PAO) and the recently discovered, spermine oxidase (SMO) [21]. Subsequent studies from our laboratory demonstrated that platinum drugs impact the polyamine biosynthetic pathway enzymes as well, by down-regulating key enzyme mRNAs [37] and activities [36].

The anti-metabolite 5FU is a cytotoxic agent that has more than one mechanism of action. After conversion to 5-fluorouridine monophosphate (FdUMP) it inhibits thymidylate synthase (TS) [30], an important enzyme in de novo DNA synthesis that converts dUMP to dTMP. 5FU can also act by direct incorporation into DNA and RNA after its activation to the corresponding nucleotide triphosphate level, resulting in DNA and RNA strand breaks and apoptosis [30]. Gene expression studies have shown that 5FU also induces SSAT gene expression in MCF7 breast cancer cells [26], HT-29 colon carcinoma cells [42], and HCT-116 colon carcinoma cells [4]. Additionally, it has been shown in HCT-116 cells that the induction of SSAT by 5FU or oxaliplatin can be enhanced when each of these agents is combined with DENSPM [9, 4] with synergistic effects on cell growth [3].

Since oxaliplatin and 5FU are used in combination in the clinic, in this study we examine the effect of adding DENSPM to this combination on SSAT and SMO mRNA, protein, enzyme activity and resulting polyamine pool levels in HCT-116 cells. We also show, for the first time, changes in SSAT and SMO gene expression patterns in tumor biopsies obtained from rectal cancer patients treated with oxaliplatin, the 5FU prodrug capecitabine and radiation.

Materials and methods

Drugs

Oxaliplatin was a gift from Dr. Paul Juniewicz of Sanofi-Synthelabo (Malvern, PA). 5FU was purchased from Sigma Chemical Company (St. Louis, MO). DENSPM was generously provided by Dr. Ronald Merriman from Pfizer Pharmaceuticals (Ann Arbor, MI).

Cell culture

The HCT-116 human colon carcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 10% Nu-Serum (BD Bioscences) and 1% L-glutamine. Cells were *mycoplasma* free and were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Drug-treatment conditions

Cells were exposed to 10 μM oxaliplatin, 10 μM 5FU or 10 μM DENSPM singly, or in the various combinations for 20 h, washed thoroughly with PBS and incubated in drug-free medium for an additional 24 h. These treatment and exposure conditions were chosen based on the fact that these are pharmacologically relevant for oxaliplatin and combining DENSPM with oxaliplatin under these conditions produced synergistic up-regulation of mRNA expression and activity for other cell lines [21, 22] and that SSAT gene expression increases with time up to 24 h after the end of drug exposure [37]. Cells were collected and assayed for gene expression, protein expression, enzyme activities and/or polyamine pools.

To examine the kinetics of induction and changes in SSAT mRNA and activity, and SMO mRNA, the cells were exposed to 10 μM oxaliplatin and 10 μM DENSPM combination for 20 h followed by incubation in drug free medium for up to 72 h. Samples were also processed immediately following drug exposure indicated as 0 h.

Quantitation of gene expression by real time quantitative RT-PCR

Real Time Quantitative RT-PCR (Taqman assay) with PE-ABI Prism 7700 Sequence Detection System was used to measure the mRNA levels of SSAT and SMO. The mRNA levels of the genes of interest and the endogenous standard β-actin were measured concurrently from the same cDNA preparations. Total RNA was extracted using Qiagen RNeasy spin columns (Qiagen, Valencia, CA) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Grand Island, NY) followed by PCR with PE-ABI 7700 (Applied Biosystems, Foster City, CA). All PCRs from each cDNA were run in triplicate. The comparative C_T method was used to quantitate gene expression [23, 25]. All mRNA expression values are ratios relative to β-actin and expressed as $\times 10^{-3}$. Fold increases were determined relative to untreated controls.

β-Actin primers and probes were purchased as ready-to-use pre-developed assay reagent (PDAR, Applied Biosystems, Foster City, CA) with a 5′ FAM (6-carboxy-fluorescein) reporter dye and 3′ TAMRA (6-carboxy-tetramethylrhodamine) quencher. SSAT primers and probes were purchased as ready to use 'Assay on Demand' kits (Applied Biosystems). SMO primers and probes were designed and purchased from Applied Biosystems under their 'Assay by Design' option. Both 'Assay on Demand' and 'Assay by Design' primers and probes are MGB (Minor Groove Binding) with a 5′ FAM reporter dye and 3′ NFQ (non-fluorescent quencher).

SSAT activity measurements

SSAT activity assay was performed as described previously [7]. In brief, the reaction mixture consisted of [^{14}C]acetyl-CoA, Spd and cell extract in Tris HCl buffer and the [^{14}C]acetylated Spd product generated by the enzyme reaction was captured on discs and subjected to radioactivity counting. The activity was expressed as pmol/min/mg protein. The total protein content in the cell lysate was determined by the Bradford assay [5].

Polyamine pools

Intracellular polyamine pools and acetylated polyamine pools were extracted with 0.6 N perchloric acid, dansylated and analyzed using reverse phase high pressure liquid chromatography with fluorescence detection as previously described [39]. Polyamine pools were expressed as pmol/10^6 cells.

Western blotting

Cells were lysed in RIPA buffer and equal amounts of total protein were loaded (SSAT 80 μg and SMO 60 μg) onto 12 and 10% SDS-PAGE gels respectively followed by transfer to polyvinylidene fluoride membrane and immunoblotted with specific antibodies. SSAT was detected as described previously [7] and SMO protein was detected using a SMO-specific antibody developed by our laboratories (Vujcic et al. unpublished). Detection was performed with enhanced chemi-luminescence reagents from Amersham Biosciences. β-Actin antibodies (Sigma, St. Louis, MO) were used to probe for internal control. Total protein was quantitated using the Bradford assay [5].

In vitro drug interaction studies

Growth inhibition assays were performed as described by Faessel et al. [14]. HCT-116 cells growing exponentially

were seeded at 800 cells/well in 96 well microtiter plates in drug free media and incubated for 24 h. Treatment with oxaliplatin and DENSPM was then carried out in fixed binary ratio mixtures (e.g. 1:4, 1:2, 1:1, 2:1, 4:1) of each drug at the predicted IC_{50} concentrations. Each of the five plates included 12 wells each for control, oxaliplatin, DENSPM and each of the five combinations of oxaliplatin and DENSPM in constant ratio with 12 dilutions. For four out of five separate experiments that were carried out, the serial dilutions were randomized as described earlier [13, 14]. After 96 h of drug exposure, the cells were fixed and stained with sulforhodamine-B (SRB), absorbance read at 570 nm and inhibition of cell growth was determined. The data were de-randomized and analyzed.

To assess the nature (synergy, additivity or antagonism) and intensity of the binary agent interactions in the combination growth inhibition studies, a simple approach adapted from work by Gessner [17] was used. The Hill model (below) is fit to each set of single agent data and each set of fixed-ratio combination concentration-effect data.

$$E = B + \frac{(Econ - B)(C/\mathrm{IC}_{50})^m}{1 + (C/\mathrm{IC}_{50})^m}$$

In this equation, E is the measured effect (absorbance); and C is dose of drug. The four estimable parameters are: *Econ*, the effect at zero concentration; B, the asymptotic effect at infinite drug concentration; IC_{50}, the median effective dose of drug; and m, a slope/sigmoidicity parameter. The Hill equation was fit to data with SAS NLIN with iteratively reweighted nonlinear regression, with the estimation of the four parameters. The weights were equal to the reciprocal of the predicted variance. Isobol graphs are then made from these data, at the IC_{10}, IC_{25}, IC_{50}, IC_{75}, and IC_{90} levels. When the isobols bow towards the Southwest corner of the graph, this indicates synergy; when the isobols bow towards the Northeast corner of the graph, this indicates antagonism; and when the isobols follow the diagonal additivity line, additivity is indicated.

SSAT and SMO gene expression in tumor biopsies from patients with rectal cancer

In a phase 1/II study, weekly doses oxaliplatin were given intravenously with daily capecitabine and radiation to patients with rectal cancer [15]. Tumor biopsies were obtained prior to therapy and 48 h after, to study the effect of this therapy on TS, TP and ERCC-1 genes (Study P.I., Marwan Fakih, MD) [15]. The study was approved by the Institutional Review Board and patients gave written informed consent for participation. With an amendment to the protocol and IRB approval, we studied the changes in SSAT and SMO gene expression caused by this therapy from the same cDNA generated to measure TS, TP and ERCC-1. SSAT and SMO gene expression measurements were carried out in tumor biopsies from 18 patients in Phase II portion of the study. The drug dosage consisted of oxaliplatin (50 mg/m^2) and capecitabine (725 mg/m^2/dose, BID, 5 days a week concurrent with radiation). Radiation therapy consisted of 1.8 GY/fraction × 28 days (Monday–Friday).

Statistical analysis

Significance of differences in mRNA levels, enzyme activity and polyamine pools in cells after drug treatments and mRNA expression from pre to post-treatment biopsies of SSAT and SMO was evaluated using Student's *t*-test and Wilcoxon signed rank Test using SigmaStat 3.1 (Systat Software Inc., Richmond, CA).

Results

The effects of adding DENSPM to oxaliplatin/5FU combination on SSAT and SMO at mRNA and protein levels and also at enzyme activity level for SSAT were examined in HCT-116 cells. Concurrently, we evaluated the effect of the single agents and two drug combinations under the same experimental conditions. Figure 1a shows that oxaliplatin/5FU/DENSPM combination produced the highest increase of SSAT mRNA compared to oxaliplatin/5FU or any of the other two drug combinations ($P < 0.01$ for all). Oxaliplatin by itself is an effective inducer of SSAT mRNA (4-fold) compared to 5FU or DENSPM (1.3- and 0.9-fold, respectively) in this cell line ($P < 0.01$). When cells were treated with oxaliplatin in combination with either 5FU or DENSPM, SSAT mRNA was up-regulated to a higher extent (5.1- and 5.9-fold, respectively) than with oxaliplatin alone ($P < 0.01$). By comparison, 5FU/DENSPM combination resulted in only a 3.1-fold increase in SSAT mRNA, but still representing a significant increase compared to the untreated control or 5FU alone ($P < 0.001$).

Although oxaliplatin/5FU combination induced significant levels of SSAT mRNA, it is apparently not translated to protein (Fig. 1b). However, adding DENSPM to this combination induced SSAT protein expression significantly. Oxaliplatin/5FU/DENSPM and Oxaliplatin/DENSPM produced similar levels of SSAT protein expression of 7.3 and 7.6-fold, respectively, relative to DENSPM alone treatment, which induced a small but discernible level of SSAT protein expression in this cell

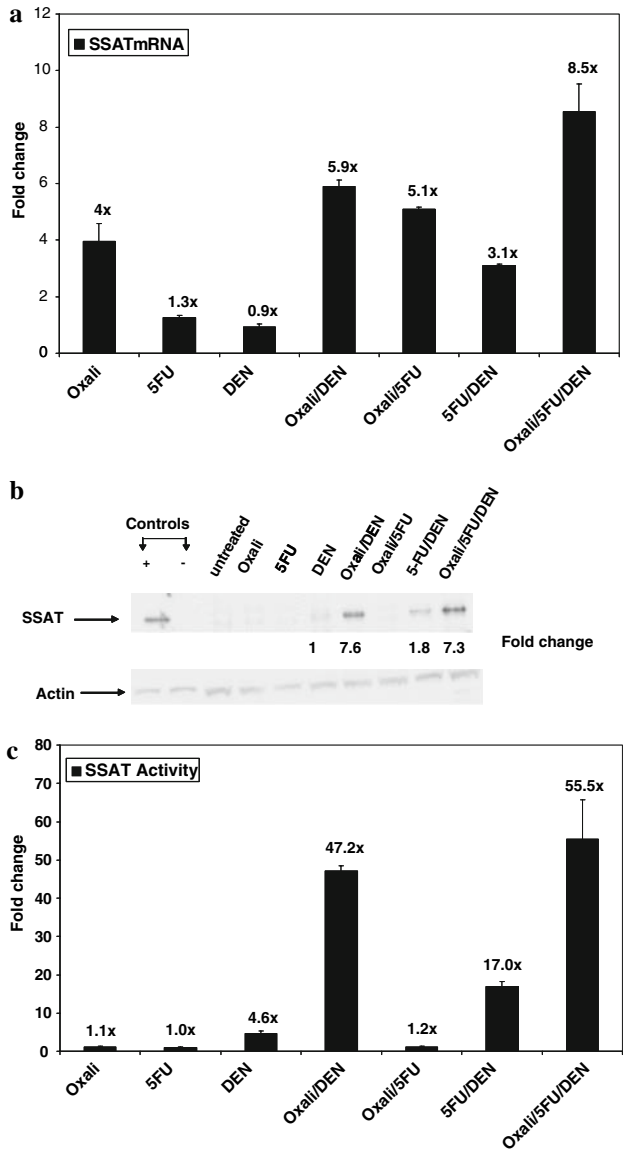

Fig. 1 Effect of oxaliplatin, 5FU and DENSPM alone and in combination on: **a** SSAT mRNA levels, **b** SSAT protein levels and **c** SSAT activity in HCT-116 colon carcinoma cells. Cells were exposed to 10 μM oxaliplatin (oxali), 5FU or DENSPM (DEN) singly, or in combination for 20 h followed by a 24 h incubation in drug-free medium. In Panel A, SSAT mRNA relative to β-actin in untreated control cells was 69.3 ± 2.8. The fold-change of the different treatment groups are shown after three independent experiments with triplicate QRT-PCR measurements. Fold changes are relative to untreated controls. In Panel B, protein levels of SSAT as quantified by densitometric measurements are shown for each treatment group from one representative Western blot out of three separate experiments. The fold change presented is in comparison to SSAT protein levels induced by DENSPM treatment. β-Actin was used as the loading control. HEK293 cell lysates treated with DENSPM was used as the positive control. In Panel C, SSAT activity of untreated cells was 169 ± 25.1 pmol/min/mg protein. Fold increase values are relative to untreated controls

line. Adding DENSPM to 5FU also produced a small increase of 1.8-fold, but single agent oxaliplatin or 5FU did not induce any SSAT protein expression. Thus adding DENSPM to the single agents oxaliplatin or 5FU or a combination of oxaliplatin/5FU, appear to facilitate the translation of SSAT mRNA.

Oxaliplatin/5FU treatment did not produce an increase in SSAT enzyme activity (Fig. 1c). The combination of DENSPM with oxaliplatin/5FU, on the other hand, produced the highest increase in SSAT activity of 56-fold relative to controls ($P < 0.001$). Adding DENSPM to oxaliplatin or 5FU also produced significant increases in SSAT activity (47.2-fold and 17-fold, respectively) relative to the no-drug controls ($P < 0.001$). Oxaliplatin/5FU/DENSPM induced significantly high levels of SSAT activity compared to oxaliplatin/5FU or 5FU/DENSPM ($P < 0.02$), but was not statistically different compared to oxaliplatin/DENSPM. Similar to SSAT protein, the single agents oxaliplatin or 5FU did not produce any significant increases in SSAT activity. Although no measurable increase in the mRNA was seen with DENSPM alone, the analog produced a 4.6-fold increase in enzyme activity relative to no-drug control ($P = 0.009$) presumably by enhancing the mRNA translation. The SSAT enzyme activity measurements reported here are the first ever with these drug combinations.

We have previously shown in A2780 ovarian carcinoma cell lines that oxaliplatin is a more effective inducer of SMO mRNA than DENSPM and the combination of oxaliplatin and DENSPM resulted in a greater than additive SMO gene expression [21]. Although SSAT mRNA and protein induction were reported, there are no published reports on SMO induction either at the mRNA or protein level with any of the combinations of oxaliplatin, 5FU or DENSPM. Considering that SMO is an important catabolic enzyme in the polyamine pathway, here we analyzed the effect of combining DENSPM with oxaliplatin/5FU along with examination of the single agents and the 2-drug combinations on SMO mRNA and protein levels in HCT-116 colon carcinoma cells (Fig. 2a,b). Oxaliplatin/5FU/DENSPM combination produced significantly higher levels of SMO mRNA compared to oxaliplatin/5FU ($P < 0.001$) (Fig. 2a). Single agents oxaliplatin, 5FU and DENSPM induced SMO mRNA to a similar extent (2.3, 1.8 and 1.8-fold, respectively) which were significant compared to the no-drug controls ($P < 0.01$). However, when DENSPM was combined with either oxaliplatin or 5FU, a >4-fold induction of SMO mRNA was observed which was greater than those seen with single agents or the oxaliplatin/5FU combination (2.3-fold) ($P < 0.001$). All DENSPM

containing combinations produced significantly higher SMO mRNA compared to any single agent or non-DENSPM combinations ($P < 0.001$).

Unlike with SSAT, SMO protein increased with all the treatments (Fig. 2b). Oxaliplatin/5FU/DENSPM induced the highest increase in SMO protein which is significantly higher than that produced by oxaliplatin/5FU. Of the single agents, DENSPM treatment resulted in the maximum increase in SMO protein levels (7.9-fold) compared to 4- and 2.7-fold for single drug treatment with oxaliplatin or 5FU, respectively. All DENSPM containing combinations produced significantly high levels of SMO protein compared to the single agents DENSPM, oxaliplatin or 5FU or the combination of oxaliplatin/5FU. These results suggest that the effects of the combinations on SMO protein levels were similar to those seen with mRNA.

As enzymes mediating polyamine catabolism, SSAT and SMO have critical role in the depletion of polyamine pools. The three drug combination of oxaliplatin/5FU/DENSPM induced the most significant depletion of spermine and spermidine pools compared to any other treatment including oxaliplatin/5FU, oxaliplatin/DENSPM and 5FU/DENSPM ($P < 0.05$ for all) in HCT-116 cells (Fig. 3a). The two drug combinations (for both spermine and spermidine pool depletions) in the order of their effectiveness were oxaliplatin/DENSPM > 5FU/DENSPM > oxaliplatin/5FU ($P < 0.05$ for all). The DENSPM combinations also produced increases in acetylated polyamine levels, especially acetylated spermidine and produced increases in putrescine levels, presumably by back conversion through polyamine oxidase (PAO). Figure 3b shows that DENSPM levels, as determined during polyamine pool analysis, were similar under all treatment conditions indicating that the co-administration of oxaliplatin or 5FU did not affect analog transport.

While the three drug combination was the most effective for SSAT and SMO induction and spermine/spermidine pool depletion, oxaliplatin/DENSPM is the only two drug combination that came close to the 3-drug activity. For this reason, we chose to perform extensive combination

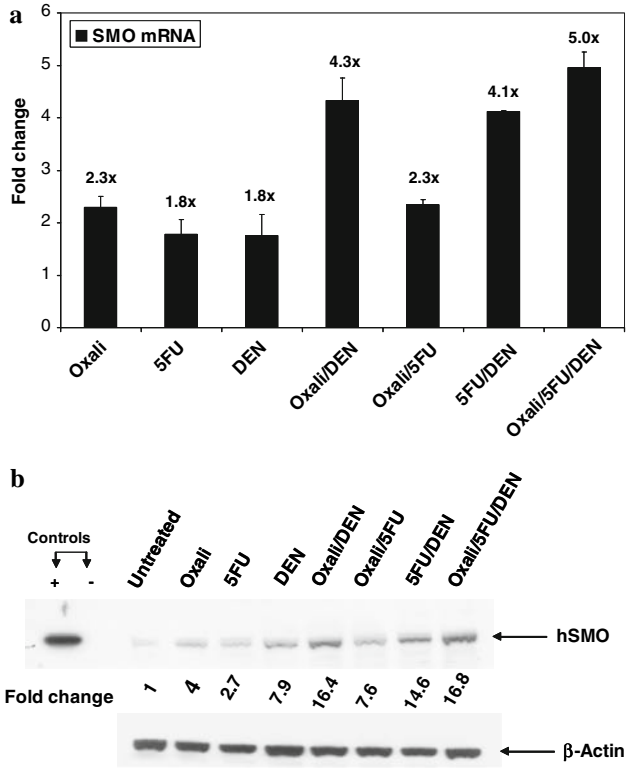

Fig. 2 Effect of oxaliplatin, 5FU and DENSPM alone and in combination on SMO **a** mRNA levels and **b** protein levels in HCT-116 colon carcinoma cells. Cells were exposed to 10 μM oxaliplatin (oxali), 10 μM 5FU or 10 μM DENSPM (DEN) singly, or in combination for 20 h followed by a 24 h incubation in drug free medium. In Panel A, SMO mRNA in untreated controls was 12.6 ± 0.54. The fold-change of the different treatment groups are shown after three independent experiments with triplicate QRT-PCR measurements. Fold changes are relative to untreated controls. In Panel B, protein levels of SSAT as quantified by densitometric measurements are shown for each treatment group from one representative Western blot out of three separate experiments. The fold changes were relative to protein levels in untreated cells in untreated cells. β-Actin was used as the loading control and over-expressed hSMO from HEK 293 cells was used as the positive control

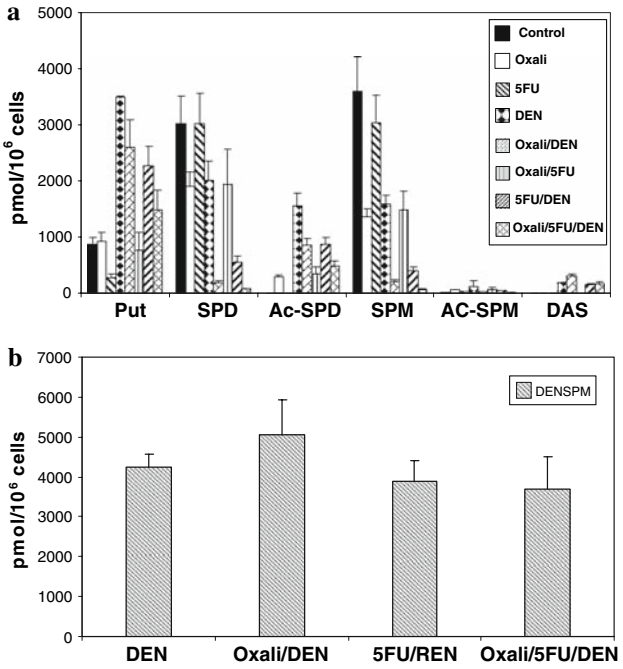

Fig. 3 Effect of oxaliplatin, 5FU and DENSPM on intracellular (**a**) polyamine pools and (**b**) DENSPM accumulation. Cells were exposed to 10 μM oxaliplatin (oxali), 10 μM 5FU or 10 μM DENSPM (DEN) singly, or in combination for 20 h followed by a 24 h incubation in drug-free medium. Values presented are mean ± SE from three independent experiments

cytotoxicity studies with oxaliplatin and DENSPM at a variety of concentrations to determine whether the drug effects on cell growth were synergistic, additive or antagonistic interactions. The results from a representative experiment are shown in Fig. 4. The drug interactions were antagonistic at IC_{10} and IC_{25}, additive at IC_{50} and synergistic at IC_{75} and IC_{90} (Fig. 4). This general pattern was also observed in four additional replicate experiments. This transition from antagonism to synergism may have important implications, as synergy at IC_{90} at which most of the cells in the tumor are expected to be killed would be of better prognostic value.

Whether the in vitro observations of induction of SSAT and SMO following oxaliplatin or 5FU occur in a clinical setting was evaluated in tumor biopsies from patients with rectal cancer in a phase II portion of a study designed to test the combined efficacy of oxaliplatin, the 5FU pro-drug capecitabine and radiation. Induction of SSAT and SMO expression was evaluated in tumor biopsies obtained prior to and 48 h following chemo-radiation. Figure 5 shows the change (i.e., the ratio of post/pre-treatment) in SSAT and SMO gene expression in rectal cancer biopsies in 18 patients following chemo-radiation. The basal level has been normalized, represented by the horizontal line at 1. Thus, bars >1 represent an increase in expression and <1 represent a decrease. Following chemo-radiation, 6/18 showed an increase in SSAT mRNA and 11 of 18 patients showed an increase in SMO mRNA. The increase in SMO from pre-treatment level was statistically significant in this group of patients ($P = 0.03$). Modest decreases in expression were also noted, mostly for SSAT. These observations raised the question as to whether these effects were dependent on the timing of the biopsy acquisition since most of the in vitro observations were made at 24 h post-drug-treatment. To address this question, we examined the induction and decay profiles for SSAT and SMO expression following the drug exposure.

On the basis of significant induction of both SSAT and SMO mRNA and protein and SSAT activity, we treated HCT-116 cells with the combination of 10 μM oxaliplatin and 10 μM DENSPM for 20 h and then examined the levels of SSAT mRNA (Fig. 6a), SSAT activity (Fig. 6b) and SMO mRNA (Fig. 6c) at 0, 24, 48 and 72 h following drug exposure. SSAT mRNA and activity both increased immediately (0 h) following a 20 h exposure to the drug combination, and reached a maximum at 24 h post-treatment. After 24 h, both mRNA and activity of SSAT have declined in parallel, showing a significant decrease at both the 48 and 72 h time. In contrast to SSAT, SMO mRNA remained stable for 72 h following the drug treatment.

Discussion

We previously reported that exposure of A2780 ovarian carcinoma cells to clinically relevant concentrations of oxaliplatin results in the potent induction of SSAT mRNA and that when combined with the polyamine analog DENSPM, there was a massive induction of SSAT mRNA and activity, together with significant depletion of polyamine pools [21]. Further, we also demonstrated that either oxaliplatin alone or in combination with DENSPM resulted in the up-regulation of mRNA for the polyamine-directed oxidases PAO and SMO, with oxaliplatin/DENSPM combination resulting in a greater than additive up-regulation for SMO compared to either of the single agents [21].

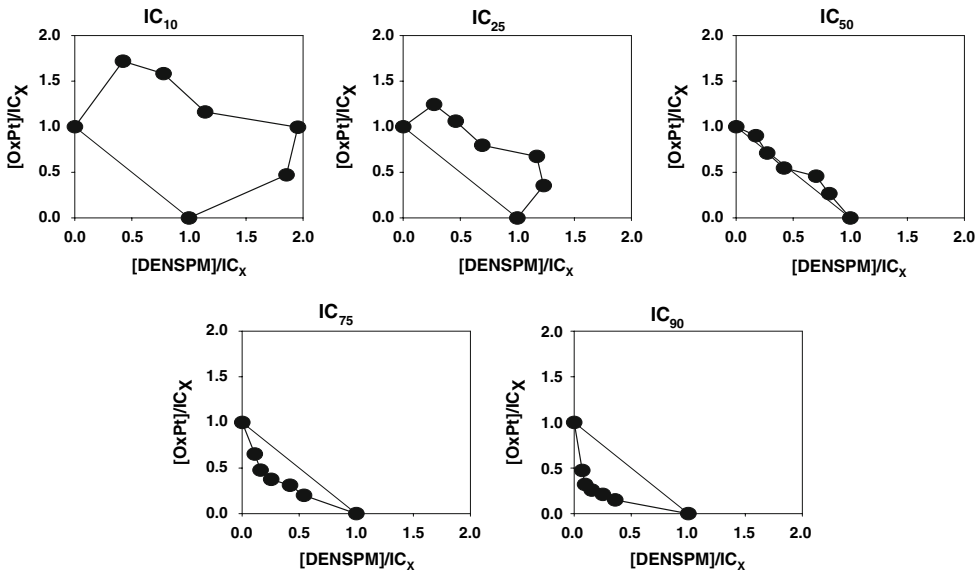

Fig. 4 Isobolograms of oxaliplatin and DENSPM in combination. The *diagonal line* represents additivity and the *curve* above the line implies antagonism as seen with the IC_{10} and IC_{25} drug concentrations. The curve below the line implies synergism as seen with the IC_{75} and IC_{90} concentrations. In the axis legends the 'x' refers to the IC concentration for each of the drugs

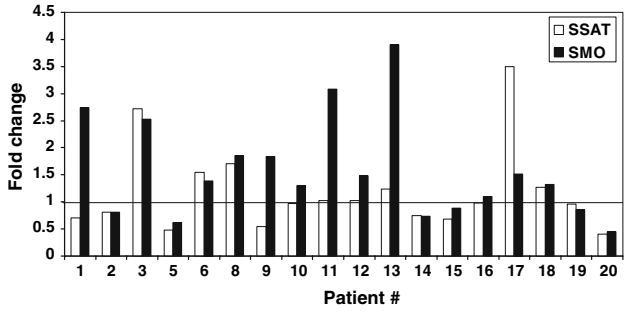

Fig. 5 Change in SSAT and SMO mRNA levels in biopsies of rectal cancer patients treated with oxaliplatin, 5FU and radiation as determined by real time QRT-PCR. Values represent fold-change determined from the ratios of post-treatment/pre-treatment mRNA levels

Recent gene expression profiling studies have highlighted the ability of various chemotherapeutic agents to induce the expression of SSAT in colon carcinoma cell lines [42, 9, 4]. This was demonstrated in HT-29 colon carcinoma cells following treatment with 5FU [42]. Additional work by this group demonstrated that 5FU induces the expression of SSAT in HCT-116 colon carcinoma cells, and that in similarity to our studies, this induction was augmented by concurrent treatment with DENSPM and produced synergistic effects on apoptosis [9]. We observed in HCT-8 colon carcinoma cells that the oxaliplatin/DENSPM combination was much more potent at inducing SSAT activity than either drug alone. Treatment with oxaliplatin or 5FU induced SSAT mRNA in a panel of colorectal cancer cell lines [3, 4], and the induction did not translate into increases in SSAT protein unless DENSPM was present [3].

The current report, unlike any of these other studies, evaluates not only the two drug combinations, but also all the 3-drugs together for their effects on polyamine catabolism. Unlike the previous reports that had only SSAT protein as the endpoint, here, in addition to protein we evaluated SSAT activity as the determinant of polyamine pool levels. As seen from the results, the 3-drug combination is most effective in inducing SSAT activity and in depleting spermine and spermidine pools. While oxaliplatin/5FU combination produced significantly higher levels of SSAT mRNA compared to the single agents, the mRNA did not translate into protein or activity and the combination did not produce significant polyamine pool depletion. The DENSPM combination with oxaliplatin was most effective in the 2-drug combinations for both SSAT protein and activity. DENSPM itself produced only a 4.6-fold increase in SSAT activity in these cells, as seen earlier in A2780 ovarian carcinoma cells with only a 4.5-fold increase and in HCT-8 colon cells with 3.5-fold increase [21, 22], perhaps a reflection of the biology of each of these cell lines.

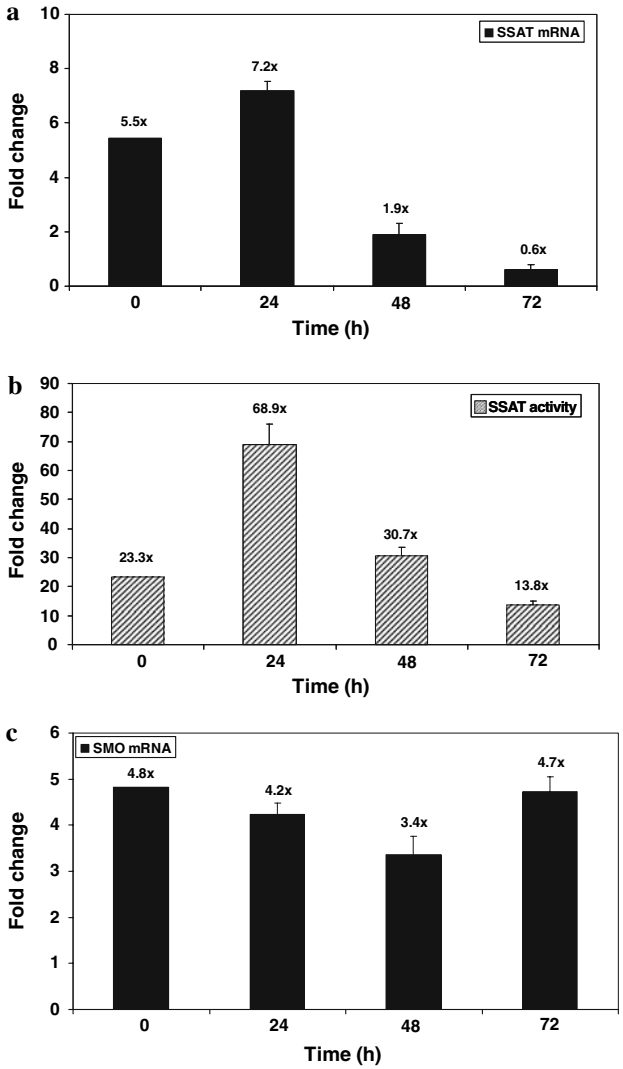

Fig. 6 Kinetics of SSAT **a** mRNA induction and **b** activity during treatment and stability following treatment in HCT-116 cells. Cells were exposed to 10 μM oxaliplatin (oxali) and 10 μM DENSPM *(DEN)* combination for 20 h followed by incubation in drug-free medium for 72 h. Samples taken immediately following drug exposure are indicated by 0 h. In **a**, SSAT mRNA levels (relative to *β*-actin) in untreated control cells was 175.9 ± 34.4. In **b**, SSAT activity in untreated cells was 175.0 ± 20.6 pmol/min/mg protein. Fold increase values represent the ratio of treated to untreated similarly grown in drug-free media. In **c**, kinetics of SMO mRNA induction in treated HCT-116 cells and changes with time. Cells were the same as in panel A and SMO mRNA levels in untreated control relative to *β*-actin was 12.2 ± 2.2

We report here, for the first time, the impact of these drug combinations on mRNA and protein for SMO, the other polyamine catabolic enzyme which directly converts Spermine to Spermidine, bypassing the sequential actions of SSAT and PAO [38, 31, 6]. Compared to all the other drugs and combinations, the 3-drug combination induced the highest levels of SMO mRNA and expression of SMO protein. In comparison to SSAT protein expression which

was induced only by DENSPM combination, SMO mRNA and protein expression were inducible to different degrees by all the treatments. However, DENSPM containing combinations were most effective in inducing both SMO mRNA and protein.

It is to be noted that the observed up-regulation of mRNA, protein or activity for SSAT and SMO with DENSPM combinations is not due to increased uptake of DENSPM into cells. While it is unclear what the underlying mechanisms are, we can only speculate that perhaps down stream effects such as inactivation of IκB contributing to transcriptional activation of SSAT [10] may be involved. As we noted before in our other studies, platinum drugs in general are potent inducers of SSAT gene expression [21] and DENSPM seems to facilitate SSAT translation [29] and protein stabilization [11].

Induction of SSAT puts in motion other biochemical and metabolic mechanisms that may contribute to cell growth inhibition. Polyamines in the cell bind to DNA and stabilize it, and their acetylation by SSAT reduces the net charge of Spermidine and Spermine by one and their ability to bind DNA [16]. The enhanced SSAT activity and subsequent polyamine pool depletion by the oxaliplatin/5FU/DENSPM combination may provide a mechanism by which Pt drugs are able to more readily gain access to DNA and thus, form more of the Pt-DNA adducts that underlie the cytotoxic effects of oxaliplatin. Previous studies have demonstrated that pre-treatment of human brain tumor cell lines with the polyamine analogues DENSPM and BE-4444 (1,19-bis(ethylamino)-5,10,15-triazanonadecane) increased the incorporation of platinum into the linker regions of DNA and enhanced the cytotoxicity of cisplatin [28]. The effects of the oxaliplatin/DENSPM combination on Pt-DNA adduct formation is an important area that needs to be further examined.

We have previously shown that cell growth inhibition of A2780 ovarian carcinoma cells can be enhanced by adding DENSPM to oxaliplatin treatment. Studies from other laboratories have demonstrated synergy between cisplatin and DENSPM in murine L1210 leukemia and B16F1 melanoma cells in both in vitro and in vivo systems [19]. More recent studies have reported synergism of oxaliplatin and DENSPM or 5FU and DENSPM at only 3 fixed concentrations of each of the drugs [3]. Although the 3-drug combination was the most effective in polyamine catabolism, since synergism and antagonism between 3-drugs together is difficult to assess, we chose the combination of oxaliplatin and DENSPM for an extensive assessment of growth effects, for the reason that it produced effects only second to the oxaliplatin/5FU/DENSPM combination in all aspects of polyamine catabolism including SSAT induction at all levels, SMO mRNA/protein induction and polyamine pool depletion. Drug interactions between oxaliplatin and DENSPM were studied at a wide range of concentrations spanning a 6 log range with different ratios of the drugs [14]. Our results indicated that oxaliplatin and DENSPM were antagonistic at IC_{10} and IC_{25}, additive at IC_{50}, synergistic at IC_{75} and highly synergistic at IC_{90}. To our knowledge, our studies are the first to extensively analyze synergism/antagonism between these drugs over a broad range of concentrations and drug combinations. It is interesting that at low concentrations there was an antagonistic effect on cell growth. While we do not know the biological basis for this, Minchin et al. [27], reported a growth enhancing effect at low concentrations of DENSPM in melanoma cells resistant to the drug. The authors hypothesized that the drug may mimic endogenous polyamines at low concentrations supporting cell growth. Our observation that the two drugs are synergistic at higher concentrations may have important implications because synergy at high concentrations can have the potential to inhibit growth or kill the last remaining cancer cell. These data suggest that clinical combinations should address strategies to maximize drug concentrations to tumor tissues and *in vivo* studies are essential to investigate some of these issues.

While many of the earlier studies on SSAT induction dealt with oxaliplatin or 5FU, there was one report that indicates SSAT induction and polyamine pool depletion also occurs following radiation in HeLa S3 cells [24]. To evaluate whether our in vitro findings of SSAT induction by oxaliplatin or 5FU could also occur in tumors of patients receiving these drugs, we performed SSAT and SMO gene expression measurements in biopsies of rectal cancer patients before and after chemo-radiation consisting of oxaliplatin and the 5FU pro-drug capecitabine. The results indicate for the first time, that chemo-radiation induced SSAT and SMO gene expression in the tumors of a certain percentage of patients, with SMO being significantly responsive than SSAT. The fewer patients exhibiting SSAT induction may have been related to non-optimal biopsy sampling times as suggested by our in vitro studies on mRNA increases and decay following the drug treatment. Further studies are needed to better understand these phenomena and to evaluate their prognostic significance.

In summary, the present study makes the novel observation that oxaliplatin/5FU/DENSPM combination is highly effective in inducing significant increases in SSAT activity, SMO protein and polyamine pool depletion. The two agents, oxaliplatin and DENSPM that appear to contribute to this 3-drug activity show synergistic cell kill at concentrations above IC_{50}. In addition, we present here for the first time that meaningful changes in SMO and SSAT mRNA levels occur during chemotherapy in rectal cancer patients receiving oxaliplatin, capecitabine and radiation. These data suggest that the combination of DENSPM,

oxaliplatin and 5FU should be evaluated and optimized in animal models as a possible prelude to clinical trials.

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