Polyamine catabolism in platinum drug action: Interactions between oxaliplatin and the polyamine analogue N^1,N^{11}-diethylnorspermine at the level of spermidine/spermine N^1-acetyltransferase

Suzanne Hector,[1] Carl W. Porter,[2] Debora L. Kramer,[2] Kimberly Clark,[1] Joshua Prey,[1] Nicholas Kisiel,[2] Paula Diegelman,[2] Ying Chen,[2] and Lakshmi Pendyala[1]

Departments of [1]Medicine and [2]Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York

Abstract

A great deal of experimental evidence connects induction of polyamine catabolism via spermidine/spermine N^1-acetyltransferase (SSAT) to antiproliferative activity and apoptosis. Following our initial observation from gene expression profiling that platinum drugs induce SSAT, we undertook this present study to characterize platinum drug induction of SSAT and other polyamine catabolic enzymes and to examine how these responses might be enhanced with the well-known inducer of SSAT and clinically relevant polyamine analogue, N^1,N^{11}-diethylnorspermine (DENSPM). The results obtained in A2780 ovarian cancer cells by real-time quantitative RT-PCR and Northern blot analysis show that a 2-hour exposure of A2780 cells to platinum drugs induces expression of SSAT, a second SSAT (SSAT-2), spermine oxidase, and polyamine oxidase in a dose-dependent manner. At equitoxic doses, oxaliplatin is more effective than cisplatin in SSAT induction. The most affected enzyme, SSAT, increased 15-fold in mRNA expression and 2-fold in enzyme activity. When combined with DENSPM to further induce SSAT and to enhance conversion of mRNA to activity, oxaliplatin increased SSAT mRNA 50-fold and activity, 210-fold. Polyamine pools declined in rough proportion to levels of SSAT induction. At pharmacologically relevant oxaliplatin exposure times (20 hours) and drug concentrations (5 to 15 μmol/L), these responses were increased even further. Combining low-dose DENSPM with oxaliplatin produced a greater than additive inhibition of cell growth based on the sulforhodamine-B assay. Taken together, the findings confirm potent induction of polyamine catabolic enzymes, such as SSAT by platinum drugs, and demonstrate that these biochemical responses as well as growth inhibition can be potentiated by co-treatment with the polyamine analogue DENSPM. With appropriate *in vitro* and *in vivo* optimization, these findings could lead to clinically relevant therapeutic strategies. [Mol Cancer Ther 2004; 3(7):813–22]

Received 12/24/03; revised 3/23/04; accepted 5/4/04.

Grant support: CA-22153, CA-76428, and CA-16056.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Lakshmi Pendyala, Department of Medicine, Roswell Park Cancer Institute, Buffalo, NY 14263. Phone: 716-845-3287; Fax: 716-845-1659. E-mail: lakshmi.pendyala@roswellpark.org

Introduction

Platinum drugs are an important component of modern chemotherapy regimens. Cisplatin (*cis*-diamminedichloro platinum II) is the prototype platinum drug showing activity in many cancers, including testicular, ovarian, head and neck, and bladder. Oxaliplatin (*trans-l*-1,2-diaminocyclohexane oxalato platinum II) is a third-generation platinum drug which in addition to exhibiting activity in tumors that are traditionally sensitive to cisplatin (1, 2) has shown activity in colon cancer, a disease in which cisplatin is inactive (3). Oxaliplatin is approved for the treatment of colorectal cancer in the United States.

Many *in vitro* studies and some *in vivo* studies indicate that oxaliplatin is non-cross-resistant to cisplatin (4-7). Comparative cytotoxicity studies of cisplatin and oxaliplatin conducted in the NCI human tumor cell line panel indicate that the two drugs may have different mechanisms of action (8). Both drugs produce intra- and inter-strand DNA-platinum adducts (7, 9, 10) that are believed to account for cytotoxicity. To gain insight into other potential mechanisms of platinum drug action, we did gene expression profiling of A2780 ovarian carcinoma cells exposed to cisplatin or oxaliplatin (11). Affymetrix oligonucleotide arrays revealed a large number of genes that were up- or down-regulated by both platinum drugs. Self-Organizing Map cluster analysis indicated that the expression changes for many genes were progressive with time and that the greatest increases or decreases occurred 16 to 24 hours following a 2-hour, IC_{90} drug exposure. Of particular interest, the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) was among the top 10 genes up-regulated by oxaliplatin and among the top 20 genes up-regulated by cisplatin. This unexpected and provocative finding warranted further investigation.

The requirement of polyamines in cell growth is typically met by a biosynthetic pathway regulated by ornithine decarboxylase and *S*-adenosylmethionine decarboxylase and balanced by a polyamine catabolic or back-conversion pathway regulated by SSAT. Because tumor cells typically contain higher polyamine levels and greater rates of

polyamine biosynthetic activity than their normal tissue counterparts (12), antitumor strategies have been developed to deplete polyamine pools. Traditionally, this has been achieved with specific inhibitors of the biosynthetic enzymes ornithine decarboxylase or *S*-adenosylmethionine decarboxylase. More recently, this has been accomplished with regulators of polyamine metabolism, such as the polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM), which down-regulates both ornithine decarboxylase and *S*-adenosylmethionine decarboxylase while potently up-regulating SSAT and polyamine catabolism (13). Several lines of evidence strongly suggest that SSAT is a key determinant of DENSPM action (14-16) and directly responsible for the differential sensitivity of various cell types to the analogue (17). DENSPM has been evaluated in phase I clinical trials (18, 19).

Polyamine catabolism or back-conversion is mediated by the sequential action of SSAT and polyamine oxidase (PAO). Thus, spermine and spermidine are acetylated by SSAT and then oxidized to spermidine and putrescine, respectively, by PAO (20). In addition, acetylated spermidine is efficiently exported out of the cells, thus, contributing to polyamine pool depletion (21). Two additional genes involved in polyamine catabolism have recently been identified: spermine oxidase (SMO) and a second SSAT (SSAT-2). SMO differs from PAO in its preference for the direct oxidation of unacetylated spermine to spermidine (22), a reaction that effectively bypasses the SSAT/PAO-mediated back-conversion pathway (23). Human SSAT-2 is located on chromosome 17 (24) and shares 46% amino acid sequence identity with SSAT that is located on the human chromosome X (25). The substrate specificity of the new enzyme is distinctly different from that of SSAT and it seems from transfection studies, that the enzyme may be isolated from intracellular polyamines by organelle compartmentalization (24). Under conditions of induction, both enzymes have the potential to impact on polyamine homeostasis and, thus, affect cell growth.

We have undertaken the current study because our earlier Affymetrix data identified SSAT induction as a prominent gene response to platinum drugs and because SSAT induction has been causally linked to growth inhibition and apoptosis (26, 27). The goals of this investigation are to further characterize the effect of platinum drugs on SSAT and other components of polyamine catabolism and to examine how these responses might be exaggerated by drug combinations involving the well-known SSAT inducer DENSPM.

Materials and Methods

Drugs

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

Cell Culture

A2780 human ovarian carcinoma cell line was a gift from Dr. Ozols (Fox Chase Cancer Center, Philadelphia, PA). The *Mycoplasma*-free cells are maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% L-glutamine and maintained at 37°C in humidified 5% CO_2 atmosphere. This cell line shows similar sensitivity to both oxaliplatin and cisplatin. The human melanoma cell lines MALME-3M and SK-MEL-28 were obtained from American Type Culture Collection (Manassas, VA) and cultured as previously described (27). The transformed human kidney HEK-293 cells were obtained from Life Technologies (Gaithersburg, MD) and cultured as previously described (22).

Drug Treatment Conditions

All drug treatments were conducted on logarithmically growing cells that were plated the previous day. Initial experiments were carried out to validate the previous Affymetrix data (11). For these, cells (1×10^6/25 cm^2 culture flask) were exposed to IC_{10} to IC_{90} concentrations of either oxaliplatin or cisplatin for 2 hours, and incubated for 24 hours in drug-free medium. Control cultures were identically manipulated but, in the absence of drug. To study the effects on gene expression, activity, and polyamine pools, 5×10^6 cells/175 cm^2 culture flask were exposed to the drug(s). For sequential drug treatments, cells were exposed for 2 hours to oxaliplatin (32 μmol/L), washed thoroughly with PBS, and incubated in drug-free or in 10 μmol/L DENSPM-containing medium for 24 hours. DENSPM treatment alone consisted of incubating cells in drug-free medium for 2 hours, followed by incubation in 10 μmol/L DENSPM for 24 hours. For concurrent drug treatments, cells were exposed to 32 μmol/L oxaliplatin and 10 μmol/L DENSPM for 2 hours, washed free of the drugs, and re-incubated for a further 24 hours in DENSPM. Cells were harvested at the end of each of the two treatments and assayed for gene expression, enzyme activity, and polyamine pools as described below. SSAT induction and polyamine pool depletion were also examined during treatment with pharmacologically relevant exposure time and concentrations of oxaliplatin. Thus, cells were treated with 5, 10, and 15 μmol/L oxaliplatin for 20 hours, cells washed free of the drug, and incubated for another 24 hours before harvesting the cells. For evaluating the combined effect with DENSPM, cells were treated concurrently with 10 μmol/L oxaliplatin and 10 μmol/L DENSPM for 20 hours, followed by a further 24 hours in drug-free medium; single agent treatments were carried out under identical exposure and further incubation conditions.

Cell Growth

Growth inhibition experiments were carried out using sulforhodamine-B micro-culture colorimetric assay as previously described (5). Sulforhodamine-B assay is a colorimetric assay in which the protein biomass is determined by dye binding to basic amino acids (28). In sequential drug treatment, cells were first treated for 2 hours with oxaliplatin, washed free of drug, and then incubated in DENSPM-containing medium (at the concentrations

indicated) for an additional 24 hours. In concurrent drug treatment, cells were exposed to varying concentrations of oxaliplatin and 0.5 μmol/L DENSPM for 2 hours, washed free of the drugs, and then incubated in 0.5 μmol/L DENSPM-containing medium for an additional 24 hours. Following either sequential or concurrent drug treatments, cells were incubated in drug-free medium for a further 48 hours, before fixation and staining with sulforhodamine-B.

Northern Blot Analysis

Northern blot analysis was used in initial experiments to evaluate the effect of oxaliplatin on the expression of both the heteronuclear mRNA (3.5 kb) and the processed mature mRNA (1.3 kb), as described previously (29). 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 nylon membrane. The membrane was hybridized to ^{32}P-labeled cDNA probes for detection of SSAT mRNA and exposed for autoradiography. The glyceraldehyde-3-phosphate dehydrogenase signal was used as a loading control.

Real-time Quantitative RT-PCR

Real-time quantitative RT-PCR (QRT-PCR; Taqman assay) with PE-ABI Prism 7700 Sequence Detection System was used to measure the expression of SSAT, SSAT-2, SMO, and PAO. The mRNA levels of the gene of interest and that of the internal standard (β-actin) were measured concurrently from the same cDNA preparations. Total RNA was extracted using Qiagen RNeasy spin columns (Qiagen, Valencia, CA). cDNA was synthesized using Superscript II reverse transcriptase followed by PCR with PE-ABI 7700 (Foster City, CA). The detection and quantitation of PCR product with PE-ABI 7700 is by fluorescence with the use of gene specific primers and a fluorogenic probe. The comparative C_T method of quantitation was used as described previously (5). All mRNA expression values are ratios to β-actin and all values are $\times 10^{-3}$. Data shown are fold increase for treated relative to untreated controls.

The primers and probes for SSAT, SSAT-2, and β-actin were purchased from Applied Biosystems Inc. (Foster City, CA), as ready-to-use kits (SSAT, Assay on Demand, Assay no. Hs00161511_m1; SSAT-2, Assay-on-Demand, Assay no. Hs00374138_g1; β-actin, pre-developed assay reagent, part no. 4310881E). The SSAT probe lies on the exon 3/exon 4 junction (accession no. NM_002970). The SSAT-2 probe lies on the exon 2/exon 3 junction (accession no. AF348524). Primers and probes for SMO and PAO were designed through Applied Biosystems Inc., under ''Assay-by-Design'' option and are as follows:

SMO (accession no. AK000753)
Forward primer: 5′-GGCAGTGGCCGAGATCTG-3′
Reverse primer: 5′-CGCCGAGGTTTTGGAATGTT-3′
Probe: 5′-FAM-TTCACAGGGAACCCC-NFQ-3′
PAO (accession no. XM_113593)
Forward primer: 5′-GGTTCCGGAAGCTCATTGG-3′
Reverse primer: 5′-GGCAATGAACCCACAGAGAAC-3′
Probe: 5′-FAM-TGGACAGACGCAAAGG-NFQ-3′
(Reverse)

Both Assay on Demand and Assay by Design probes are MGB (Minor Groove Binding) with a 5′ FAM (6-carboxy-fluorescein) reporter dye and 3′ NFQ (non-fluorescent quencher).

SSAT Activity

SSAT activity assay was done as described previously (17). In brief, the reaction mixture consists of [^{14}C]acetyl-CoA, spermidine, and cell extract in Tris-HCl buffer and the [^{14}C]acetylated spermidine product generated by the enzyme reaction is captured on discs and subjected to radioactivity counting. The activity is expressed as pmol/min/mg protein.

Polyamine Pools

Intracellular polyamine pools and acetylated polyamine pools were extracted with 0.6 N perchloric acid, dansylated and analyzed using reverse phase high-performance liquid chromatography with fluorescence detection as previously described (16).

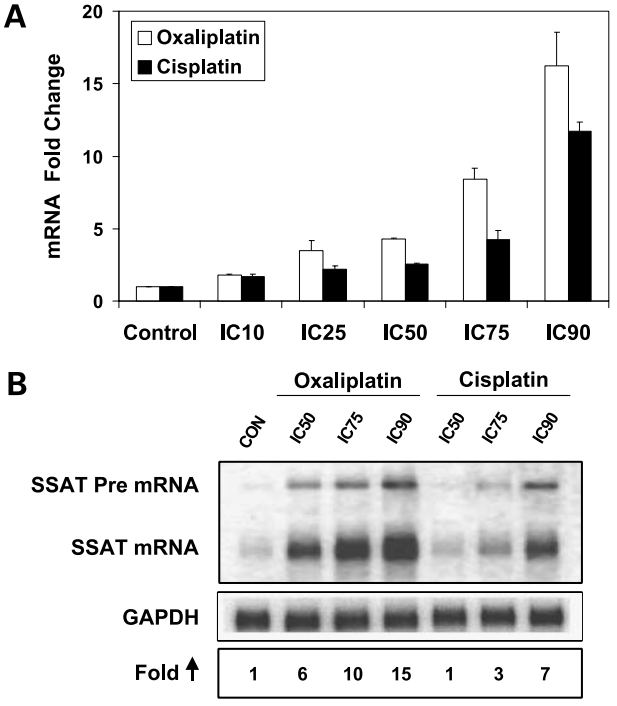

Figure 1. Effect of platinum drugs on SSAT mRNA. **A,** changes in SSAT mRNA in A2780 cells following exposure to increasing concentrations of either oxaliplatin or cisplatin, measured by real-time QRT-PCR. Cells were treated for 2 hours with IC_{10}, IC_{25}, IC_{50}, IC_{75}, and IC_{90} concentrations of oxaliplatin at 2.8, 4.8, 8.5, 17, and 32 μmol/L, respectively, or cisplatin at 2.6, 4.0, 6.4, 12, and 25 μmol/L, respectively, followed by 24 hours incubation in drug-free medium before RNA extraction. SSAT/β-actin mRNA in control cells was 2.7 ± 0.1. *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE. **B,** Northern blot analysis of A2780 cells exposed to oxaliplatin versus cisplatin. Cells were treated for 2 hours with oxaliplatin or cisplatin at IC_{50}, IC_{75}, and IC_{90} concentrations followed by 24 hours incubation in drug-free medium before RNA extraction and hybridization. SSAT mRNA bands were scanned densitometrically, normalized to glyceraldehyde-3-phosphate dehydrogenase, and expressed as fold increase for treated relative to control (*CON*). The data show that the heteronuclear (*Pre mRNA*, ~3.5 kb) as well as the mature message (~1.3 kb) increased with treatment.

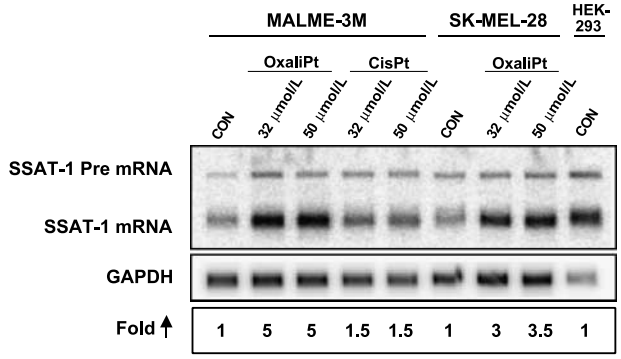

Figure 2. Effect of oxaliplatin or cisplatin on SSAT-1 mRNA expression in MALME-3M and SK-MEL-28 cells as evaluated by Northern blot analysis. Cells were treated for 2 hours with oxaliplatin (*OxaliPt*) or cisplatin (*CisPt*) at 32 or 50 μmol/L and then incubated in drug-free medium for an additional 24 hours before RNA isolation (10 μg total RNA loaded per lane). Data are representative of findings from two experiments.

Results

The changes in SSAT gene expression previously noted by Affymetrix analysis were evaluated by real-time QRT-PCR after a 2-hour exposure of cells to IC_{10} to IC_{90} concentration of oxaliplatin or cisplatin followed by a 24-hour incubation in drug-free medium. As shown in Fig. 1A, there was a dose-dependent increase in the SSAT expression following treatment with either platinum drug. At equitoxic concentrations, oxaliplatin was more effective at increasing SSAT mRNA than cisplatin. Oxaliplatin increased expression 2-fold at an IC_{10} concentration and ~16-fold at an IC_{90} dose.

Northern blot analysis was carried out on oxaliplatin- and cisplatin-treated A2780 cells (Fig. 1B). A major increase in mature SSAT mRNA (~1.3 kb) was accompanied by a concomitant increases in heteronuclear pre-processed SSAT mRNA (~3.5 kb), suggesting an increase in SSAT gene transcription (30). The increases in both the heteronuclear and mature SSAT mRNA were concentration dependent and confirm the QRT-PCR observation, that at equitoxic concentrations, oxaliplatin is a more potent inducer of SSAT than cisplatin. To determine whether SSAT induction by platinum drugs was unique to A2780 cells, induction of SSAT was examined in MALME-3M and SK-MEL-28 human melanoma cell lines. Northern blot analysis (Fig. 2) revealed that in similarity to A2780 cells, oxaliplatin induced SSAT expression by 3- to 5-fold in these cell lines.

Although the IC_{90} of oxaliplatin produced a 15-fold increase in SSAT mRNA, SSAT activity rose by only ~2-fold (Fig. 3A). This is consistent with a known translational control of SSAT message that limits induction of SSAT activity (30). As shown in Fig. 3B, this modest increase in activity was associated with a ~30% reduction in the cellular spermine, 40% decrease in spermidine, and 60% decrease in putrescine. Despite these polyamine changes, the actual SSAT products, acetylspermine or acetylspermidine, were not detected in polyamine pool analysis.

We have previously shown that SSAT activity is under posttranscriptional regulation and that significant mRNA induction is not always followed by comparable increases in enzyme activity (30, 31). This translational block can be overcome by posttreatment with a natural polyamine, such as spermine, or even more effectively, with a polyamine analogue, such as DENSPM (30). Thus, nonspecific induction of SSAT mRNA by inhibitors of proteins synthesis, for example, could be converted to huge increases in enzyme activity by posttreatment with DENSPM (30). Following this paradigm, we first treated cells for 2 hours with 32 μmol/L oxaliplatin to transcriptionally induce SSAT mRNA and then exposed them to 10 μmol/L DENSPM for 24 hours to facilitate translation of message and stabilization of enzyme protein. The effects of this drug combination on SSAT gene expression and SSAT activity and the resulting impact on intracellular polyamine pools are shown in Fig. 4A and B. Oxaliplatin alone increased SSAT mRNA 15-fold, DENSPM increased it 5-fold, and the drug

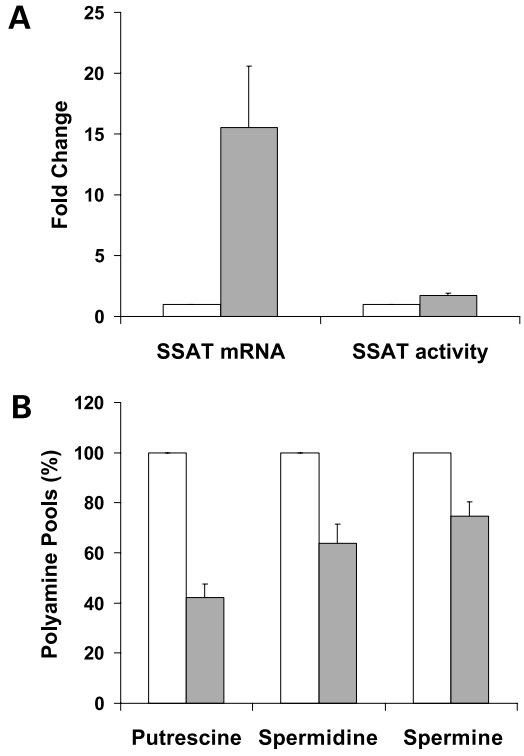

Figure 3. Effect of oxaliplatin on SSAT mRNA, activity, and polyamine pools in A2780 cells. **A,** changes in SSAT mRNA and enzyme activity in A2780 cells following exposure to 32 μmol/L oxaliplatin for 2 hours and incubation in drug-free medium for 24 hours. *Open bars*, control cells that did not receive the drug; *filled bars*, oxaliplatin-treated cells. SSAT/β-actin mRNA in control cells was 3.1 ± 0.24; SSAT enzyme activity in control cells was 29.1 ± 0.3 pmol/min/mg protein. Gene expression data: *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE. Enzyme activity data: *Columns*, mean fold change (relative to untreated controls), where $n = 3$; *bars*, SE. **B,** changes in putrescine, spermidine, and spermine pools in A2780 cells following exposure to 32 μmol/L oxaliplatin for 2 hours and incubation in drug-free medium for 24 hours. *Open bars*, control cells that did not receive the drug; *filled bars*, oxaliplatin-treated cells. *Columns*, means ($n = 3$); *bars*, SE. Putrescine in control cells was 604 ± 130 pmol/10^6 cells; spermidine, 3,704 ± 496 pmol/10^6 cells; spermine, 2,387 ± 332 pmol/10^6 cells.

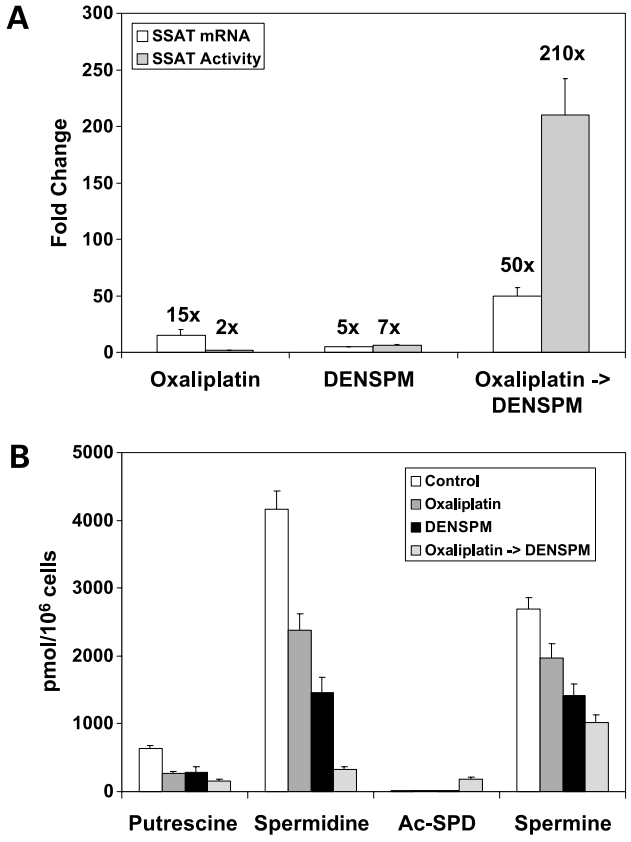

Figure 4. Effect of oxaliplatin alone (32 μmol/L oxaliplatin for 2 hours followed by 24 hours in drug-free medium), DENSPM alone (2 hours drug-free medium followed by 10 μmol/L DENSPM for 24 hours), or oxaliplatin plus DENSPM (32 μmol/L oxaliplatin for 2 hours followed by 10 μmol/L DENSPM for 24 hours) on (**A**) SSAT mRNA, SSAT activity, and (**B**) polyamine pools. In **A,** SSAT/β-actin mRNA in control cells was 3.1 ± 0.24; SSAT enzyme activity in control cells was 29.1 ± 0.3 pmol/min/mg protein. Gene expression data: *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE. Enzyme activity data: *Columns*, mean fold change (relative to untreated controls), where $n = 3$; *bars*, SE. In **B,** polyamine pools are expressed as pmol/10^6 cells ± SE, where $n = 3$. *Ac-SPD*, N^1-acetylspermidine.

combination brought a 50-fold increase. An even greater interaction between the two drugs was seen at the level of SSAT activity. Relative to the untreated control, oxaliplatin increased enzyme activity ~2-fold, DENSPM, 7-fold and the drug combination, 210-fold. We note that these fold increases in activity almost certainly underestimate the actual increase in activity because nonspecific cellular acetyltransferases detected by the assay contribute significantly to the basal enzyme levels but to a much lesser extent to the drug-induced levels (17). Consistent with the increase in SSAT activity, all the polyamine pools were markedly lowered after each of the three drug treatments (Fig. 4B). They were most affected, however, by the sequential combination, which produced a 76% depletion in putrescine, a 92% depletion of spermidine, and a 62% depletion of spermine pools. A very significant increase in N^1-acetylspermidine (i.e., from not detectable levels to 182 pmol/10^6 cells) was observed in cells treated with the drug combination, with the presumption that even more product was probably exported into the media.

We next determined whether the potent increase in SSAT mRNA and activity was affected by how the two drugs were combined. Thus, the effects of a 2-hour concurrent exposure to oxaliplatin (32 μmol/L) and DENSPM (10 μmol/L) followed by 24 hours DENSPM was compared with the sequential exposure described above. As shown in Fig. 5, the increase in SSAT mRNA and activity was significantly greater when oxaliplatin and DENSPM were administered concurrently and then followed by additional DENSPM treatment. SSAT activity rose 423-fold during concurrent treatment as compared with 210-fold during sequential treatment. Under these same conditions, near total depletion of all three polyamines was achieved (i.e., putrescine was depleted by 97%; spermidine, by 97%; and spermine, by 76%).

We next examined the effects of platinum drugs on the expression of the three recently identified polyamine catabolic enzymes: SMO (22), PAO (20), and SSAT-2 (24). As shown in Fig. 6, both platinum drugs induced all three genes in a dose-dependent manner with SMO expression being the most affected followed by PAO and SSAT-2. The effect of the drug combination on these genes was then examined (Fig. 7). In similarity to observations in other cell lines (24), DENSPM did not increase SSAT-2 mRNA levels in A2780 cells. It did, however, increase PAO and SMO, but not as effectively as oxaliplatin. Following 24 hours treatment with 10 μmol/L DENSPM, the fold change for SMO, PAO, and SSAT-2, mRNA was 2.5-, 1.4-, and 0.7-fold, respectively, compared with 7-, 3-, and 3.2-fold, respectively,

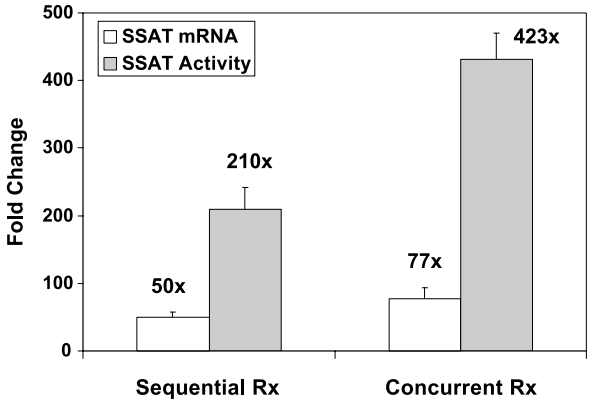

Figure 5. Effect of drug (oxaliplatin and DENSPM) exposure schedule on SSAT mRNA and activity. For sequential exposures, cells were treated with 32 μmol/L oxaliplatin for 2 hours, followed by 10 μmol/L DENSPM for 24 hours; the mRNA and activity levels in control cells are as shown in Fig. 4. For concurrent exposure, cells were treated with 32 μmol/L oxaliplatin and 10 μmol/L DENSPM for 2 hours, followed by 10 μmol/L DENSPM for 24 hours. SSAT/β-actin mRNA in control cells was 4.7 ± 0.4; SSAT enzyme activity in control cells was 24 ± 1.0 pmol/min/mg protein. Gene expression data: *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE. Enzyme activity data: *Columns*, mean fold change (relative to untreated controls), where $n = 3$; *bars*, SE.

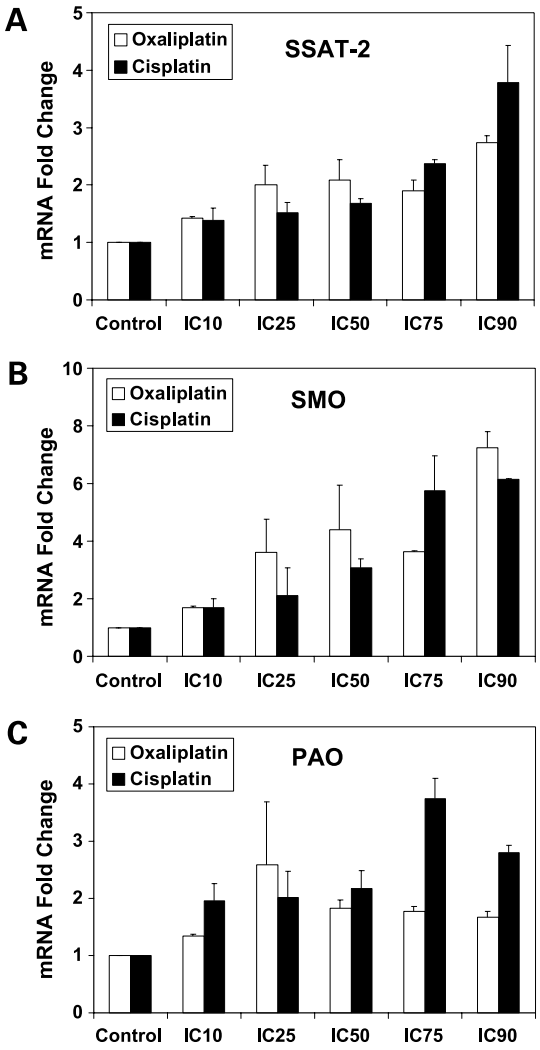

Figure 6. Changes in mRNA expression for SSAT-2, SMO, and PAO in A2780 cells following exposure to increasing concentrations of either oxaliplatin or cisplatin, measured by real-time QRT-PCR. The experimental details are the same as those described for Fig. 1. Aliquots of the same cDNA generated for SSAT mRNA expression experiments described in Fig.1A are used here for the measurement of SSAT-2 (**A**), SMO (**B**), and PAO (**C**) expression. The levels of SSAT-2, SMO, and PAO mRNA in control (no drug) were 27.5 ± 0.7, 0.9 ± 0.2, and 1.9 ± 1.0, respectively. *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE.

with 32 μmol/L oxaliplatin for 2 hours. The sequential drug combination induced significantly more SMO mRNA than either drug alone, whereas induction of PAO and SSAT-2 was similar to that of oxaliplatin alone (Fig. 7). Thus, the fold increase in expression of SMO was 2.5-fold by DENSPM alone, 7-fold by oxaliplatin alone, and 16-fold, by the drug combination. Concurrent treatment conditions yielded nearly identical effects on the three genes as sequential treatment (data not shown).

Considering that clinical pharmacokinetic studies of oxaliplatin indicate a 20-hour half-life for the free platinum and that the plasma concentrations of free and total platinum are in the order of 5 to 15 μmol/L (32, 33), we evaluated the effect of a 20-hour exposure of 5, 10, and 15 μmol/L oxaliplatin followed by a 24 incubation in drug-free medium on SSAT mRNA, activity, and polyamine pools. As shown in Fig. 8A, these pharmacologically relevant treatment conditions induced higher levels of SSAT mRNA and activity than those seen under the more intensive treatment conditions depicted in Fig. 4. Similarly, cellular spermine and spermidine pools were depleted by

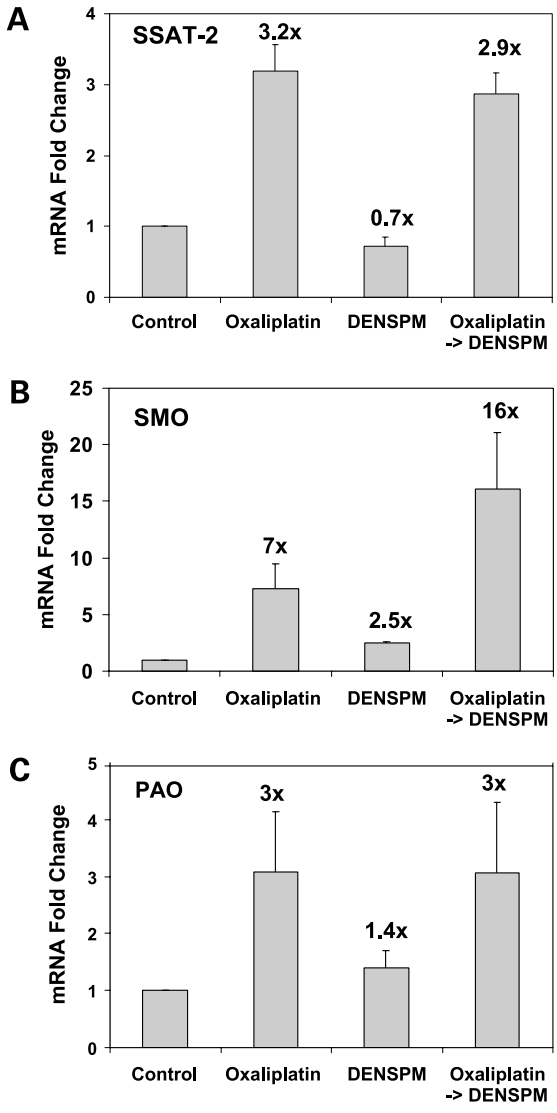

Figure 7. Effect of combining oxaliplatin with DENSPM on the expression of SSAT-2, SMO, and PAO mRNA. A2780 cells treated with oxaliplatin alone, DENSPM alone, or oxaliplatin followed by DENSPM were analyzed for levels of SSAT-2, SMO, and PAO mRNA using real-time QRT-PCR as described in Fig. 4. Aliquots of the same cDNA generated for SSAT mRNA expression experiments described in Fig. 4 are used here for the measurement of SSAT-2 (**A**), SMO (**B**), and PAO (**C**) expression. The levels for SSAT-2, SMO, and PAO mRNA in control (no drug) were 35.6 ± 7.8, 1.1 ± 0.1, and 0.9 ± 0.02, respectively. *Columns*, mean fold change (relative to untreated controls) from two separate experiments, each consisting of three separate PCRs; *bars*, SE.

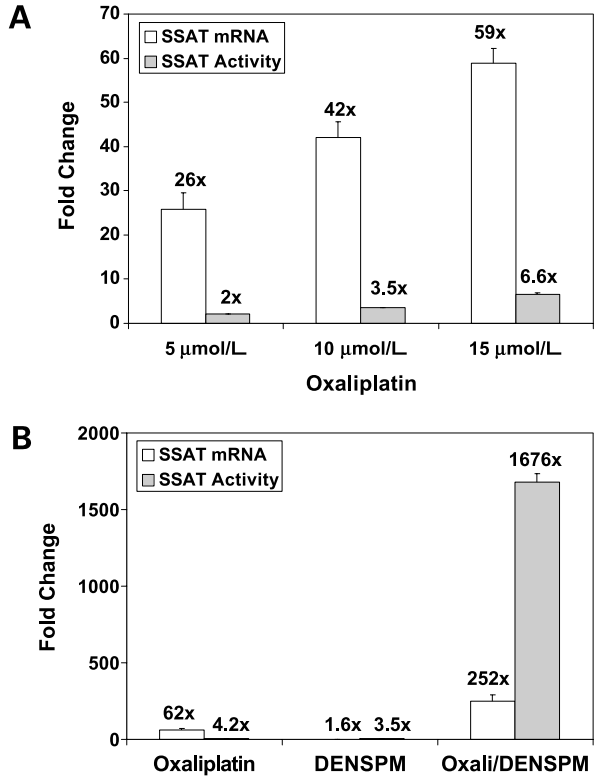

Figure 8. **A,** effects of pharmacologically relevant exposure time (20 hours) and concentrations (5, 10, or 15 μmol/L) of oxaliplatin on SSAT mRNA and enzyme activity. Cells were treated with oxaliplatin at the defined dose for 20 hours followed by a 24-hour incubation in drug-free medium, before extraction. SSAT/β-actin mRNA in control cells was 12.4 ± 0.82; SSAT enzyme activity in control cells was 23.0 ± 2.1 pmol/min/mg protein. **B,** effect of a concurrent 20 hours treatment of cells with 10 μmol/L oxaliplatin and 10 μmol/L DENSPM on SSAT mRNA and enzyme activity. Cells were treated with either drug alone or oxaliplatin and DENSPM combination followed by a 24-hour incubation in drug-free medium before extraction. SSAT/β-actin mRNA in control cells was 17.0 ± 0.68; SSAT enzyme activity in control cells was 16.9 ± 0.72 pmol/min/mg protein. **A** and **B,** gene expression data: *Columns*, mean fold change (relative to untreated controls) from three separate experiments, each consisting of six separate PCRs; *bars*, SE. Enzyme activity data: *Columns*, mean fold change (relative to untreated controls), where $n = 3$; *bars*, SE.

~80% at the highest oxaliplatin dose and there was a significant accumulation of the SSAT product, N^1-acetylspermidine. Under these same oxaliplatin treatment conditions, concurrent treatment with 10 mol/L DENSPM followed by a 24-hour incubation in drug-free medium resulted in massive 250-fold increase in SSAT mRNA and nearly 1,700-fold increase in enzyme activity (Fig. 8B). This led to a near-total depletion in cellular spermine and spermidine.

Because induction of SSAT has been convincingly linked to the antiproliferative effects of DENSPM (14-16), we sought to determine whether the substantial increases in SSAT activity seen with the drug combinations would translate into similarly enhanced effects on cell growth. A dose response of DENSPM alone for 24 hours revealed an IC_{50} of ~1 μmol/L (Fig. 9A). Because the concentration of DENSPM used in enzyme induction studies (10 μmol/L) was cytotoxic in the growth inhibition assay, we used analogue concentrations that minimally affected cell growth on their own. Thus, 0.1, 0.25, and 0.5 μmol/L DENSPM reduced growth by 0%, 10% and 20%, respectively. Figure 9B depicts the dose-response curves following 2 hours exposure of cells to oxaliplatin at concentrations ranging from 0.1 to 100 μmol/L followed by a 24-hour exposure to each of the above DENSPM concentrations and a further 48 hours in drug-free medium. When combined sequentially with 0.5 μmol/L DENSPM, the oxaliplatin-dose-response curve shifted ~1 log to the left, indicating DENSPM sensitization of the cells to oxaliplatin.

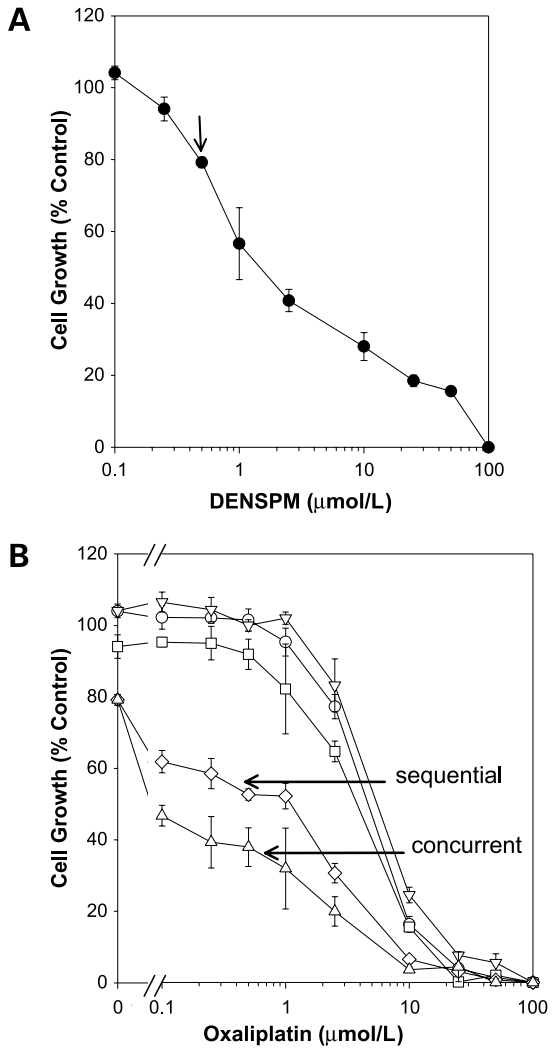

Figure 9. Growth inhibition dose-response curves to DENSPM alone (**A**) or oxaliplatin combined with low-dose DENSPM (**B**). **B,** ○, oxaliplatin; ▽, oxaliplatin (2 hours) followed by 0.1 μmol/L DENSPM for 24 hours; □, oxaliplatin (2 hours) followed by 0.25 μmol/L DENSPM for 24 hours; ◇, oxaliplatin (2 hours) followed by 0.5 μmol/L DENSPM for 24 hours; △, is concurrent 2 hours exposure to oxaliplatin and DENSPM (0.5 μmol/L) followed by 24 hours DENSPM (0.5 μmol/L). Data are percentage growth as determined by ($A_{570\ nm}$ of treated cells/$A_{570\ nm}$ of untreated cells) × 100. *Columns*, means, where $n = 3$ to 9; *bars*, SE. In **A,** the *arrow arrow* indicates the 0.5 μmol/L DENSPM with zero oxaliplatin.

Concurrent exposure to oxaliplatin and 0.5 μmol/L DENSPM followed by 24 hours DENSPM at 0.5 μmol/L shifted the curve even further to the left and, thus, produced even greater sensitization to oxaliplatin. As shown in Fig. 8B, concentrations of oxaliplatin which themselves were not growth inhibitory became growth inhibitory in combination with 0.5 μmol/L DENSPM to an extent that was greater than that produced by DENSPM alone.

Discussion

The work presented here indicates that the platinum drugs oxaliplatin and cisplatin have a profound effect on the expression of SSAT, the polyamine catabolic enzyme that acetylates spermidine and spermine and thereby promotes their degradation and/or export out of the cell. Under the conditions of these experiments, both oxaliplatin and cisplatin increased SSAT mRNA in A2780 cells more effectively than the best known inducer of SSAT gene expression, DENSPM (34). Thus, our earlier observation made with Affymetrix analysis that platinum drugs potently induce SSAT was confirmed here by real-time QRT-PCR and by Northern blot analysis. SSAT gene expression increased in a dose-dependent manner with both drugs from ~2-fold at IC_{10} up to >10-fold at IC_{90} concentrations and oxaliplatin tended to have a greater effect on SSAT gene expression than cisplatin at the equitoxic concentrations. The observation by Northern blotting that SSAT heteronuclear RNA also increased would seem to indicate that in similarity to DENSPM (29), platinum drugs induced SSAT expression by activating gene transcription, an observation that warrants further investigation.

In addition to polyamine analogues (34), a relatively large number of agents have been shown to induce SSAT activity, such as hormones, growth factors, toxic compounds, drugs, and pathophysiologic insults (35). Recently, it was shown that SSAT is the most potently induced gene from among 2,400 candidate genes in MCF-7 breast cancer cells treated with 5-flurouracil (36). Given the wide range of agents that induce SSAT, it is tempting to consider that polyamine acetylation may represent a generalized stress response. This interpretation, however, is offset by the fact that expression of other polyamine catabolic enzymes, such as SMO, PAO, and SSAT-2, is also increased, suggesting a more concerted metabolic response to the drug. It is also relevant that our earlier Affymetrix studies of platinum drug-treated cells indicated a 2-fold decline in expression of the polyamine biosynthetic enzyme, *S*-adenosylmethionine decarboxylase expression (11), although this finding has not yet been validated and explored in a manner similar to these studies with SSAT. The finding that under pharmacologically relevant treatment conditions, oxaliplatin induces both SSAT mRNA and enzyme activity and that the levels of enzyme induction are similar to those attained with DENSPM suggests that SSAT induction could represent a previously unrecognized contributor to oxaliplatin mechanism of action.

A major finding of this present study is that the platinum drug-induced SSAT mRNA can be converted to a massive increase in enzyme activity by co- or posttreatment with the polyamine analogue DENSPM. More specifically, oxaliplatin increased SSAT activity by ~2-fold, DENSPM by ~7-fold, and the combination of oxaliplatin and DENSPM by >200 or 400-fold, depending on drug combination schedule. As indication of enzyme functionality, polyamine pools were almost totally depleted due presumably to acetylation followed by either export or oxidation by PAO. The DENSPM/SSAT interaction was further enhanced when cells were concurrently exposed to oxaliplatin and DENSPM for 20 hours, treatment conditions reflecting exposures obtained during clinical studies (32, 33). The combination increased the SSAT enzyme activity by 1,676-fold, whereas as single agents, oxaliplatin and DENSPM induced a 4.2- and 3.5-fold increases in enzyme activity, respectively. Although induction of SSAT is a notoriously heterogeneous response among cell lines (37), we note that the basal SSAT enzyme activity and the polyamine pool levels in these cells are similar to those reported for other cell lines, such as MCF-7 breast cancer cells and various human melanoma cell lines (37). The finding that DENSPM enhances the oxaliplatin effect significantly is consistent with previous reports from our laboratory showing that ''super-induction'' of SSAT mRNA by inhibitors of protein synthesis resulted in minor increases in enzyme activity unless followed by treatment with polyamines or a polyamine analogue which facilitate translation and stabilization of the enzyme protein and greatly amplified the initial mRNA response at the level of SSAT activity (30, 31). Such an amplification of SSAT has not, however, been previously shown with a clinically relevant anticancer agent, such as the platinum drugs. The implications of this effect are raised by the fact that SSAT induction is causally linked to either the apoptotic or antiproliferative effects of DENSPM (14-16). Some of the more defining evidence for this relationship is as follows: (1) cells made resistant to DENSPM are unable to induce SSAT (38); (2) conditional overexpression of SSAT leads to growth inhibition in both MCF-7 breast cancer cells (26) and in LNCaP prostate carcinoma cells (39); and (3) transient transfection of cells with SSAT-directed siRNA prevents the induction of SSAT and apoptotic response (27). An obvious possibility that flows from this evidence is that this drug combination may have similarly enhanced effects on cell growth and/or cytotoxicity and, thus, therapeutic potential. Thus, using a SSAT-based rationale for combining oxaliplatin and DENSPM, we examined the effects of analogue concentrations which themselves, were minimally cytotoxic. As shown in Fig. 9, at least one such dose (0.5 μmol/L) which is known to be clinically achievable (18, 19), potentiated oxaliplatin cytotoxicity. Despite the promising nature of these findings, more detailed studies in which the drug combination is pharmacologically and mechanistically optimized are likely to produce cytotoxicity responses approaching true drug synergy. It is also relevant that induction of SSAT gene expression by platinum drugs was

not unique to A2780 cells but also occurs in additional cell types, as shown here for melanoma cells MALME-3M and SK-MEL-28. How it compares to normal cells and, thus, to drug selectivity has not yet been determined.

Synergy between DENSPM and cisplatin has been reported against murine cell lines L1210 leukemia and B16F1 melanoma both *in vitro* and *in vivo* systems (40). Synergy between N^1,N^{12}-diethylspermine (DESPM) and cisplatin was reported in cisplatin-sensitive and -resistant 2,008 ovarian carcinoma cells (41). Another study reported that pretreatment of human brain tumor cell lines with polyamine analogues, such as DENSPM, increased the incorporation of platinum into the linker regions of DNA and also the cytotoxicity of cisplatin (42). To our knowledge, only one other laboratory has reported a relationship between cisplatin and polyamine analogues that converges at SSAT. Marverti et al. (41) found that cisplatin-resistant cell lines were cross-resistant to the polyamine analogue DESPM and that there is a reduced ability by the polyamine analogue to induce SSAT in the resistant cells. Their studies further showed a reduced SSAT protein turnover following treatment with DESPM in cisplatin-sensitive cells relative to the resistant cells, indicating that cisplatin resistance modulates the SSAT response to DESPM at the transcriptional and posttranscriptional levels (43). In distinction to the present study, these investigators did not determine that cisplatin induces SSAT expression or that a polyamine analogue can potently augment this response.

In conclusion, our study showed that platinum drugs and especially oxaliplatin potently induce SSAT gene expression and that, this induction can be amplified by DENSPM by mechanisms that would seem to involve analogue-facilitated translation of the platinum-drug induced mRNA into enzyme protein. It is also clear that the platinum drug effects on polyamine catabolism are not limited to SSAT but include the enzymes SMO, PAO, and SSAT-2. We propose that with further refinement, these observations can be translated to clinical benefit. This belief is consistent with the facts that polyamine metabolism represents a validated target for therapeutic intervention, SSAT induction is known to inhibit cell growth, the potent SSAT inducer DENSPM has undergone clinical evaluation, and oxaliplatin is an important chemotherapeutic agent for treating various human cancers.

References

1. Mathe G, Kidani Y, Segiguchi M, et al. Oxalato-platinum or 1-OHP, a third-generation platinum complex: an experimental and clinical appraisal and preliminary comparison with *cis*-platinum and carboplatinum. Biomed Pharmacother 1989;43:237-50.

2. Misset JL, Kidani Y, Gastiaburu J, et al. Oxalatoplatinum (1-OHP): experimental and clinical studies. In: Howell SB, editor. Platinum and other metal coordination compounds in cancer chemotherapy. New York: Plenum Press; 1991. p. 369-75.

3. Wiseman LR, Adkins JC, Plosker GL, Goa K. Oxaliplatin. A review of its use in the management of metastatic colorectal cancer. Drugs Aging 1999;14:459-75.

4. Pendyala L, Creaven PJ. *In vitro* cytotoxicity, protein binding, red blood cell partitioning and biotransformation of oxaliplatin. Cancer Res 1993;53:5970-6.

5. Hector S, Bolanowska-Higdon W, Zdanowicz J, Hitt S, Pendyala L. *In vitro* studies on the mechanisms of oxaliplatin resistance. Cancer Chemother Pharmacol 2001;48:398-406.

6. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. Cellular and molecular pharmacology of oxaliplatin [Review] [100 refs]. Mol Cancer Ther 2002;1:227-35.

7. Di Francesco AM, Riccardi R. Cellular and molecular aspects of the drugs of the future. Cell Mol Life Sci 2002;59:1914-27.

8. Rixie O, Ortuzar W, Alvarez M, et al. Oxaliplatin, tetraplatin, cisplatin and carboplatin: spectrum of activity in drug-resistant cell lines and in the cell lines of the National Cancer Institute's anticancer drug screen panel. Biochem Pharmacol 1996;52:1855-65.

9. Woynarowski JM, Chapman WG, Napier C, Herzig MS, Juniewicz P. Sequence- and region-specificity of oxaliplatin adducts in naked and cellular DNA. Mol Pharmacol 1998;54:770-7.

10. Woynarowski JM, Faivre S, Herzig MS, et al. Oxaliplatin-induced damage of cellular DNA. Mol Pharmacol 2000;58:920-7.

11. Hector S, Hawthorn L, Greco W, Pendyala L. Gene expression profiles after oxaliplatin treatment in A2780 ovarian carcinoma cells. Proc AACR 2002;43:62.

12. Porter C, Herrera-Omelas L, Pera P, Petrelli NF, Mittleman A. Polyamine biosynthetic activity in normal and neoplastic human colorectal tissues. Cancer 1987;60:1275-81.

13. Thomas T, Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 2001;58:244-58.

14. Casero RAJ, Celano P, Ervin SJ, Porter CW, Bergeron RJ, Libby PR. Differential induction of spermidine/spermine N^1-acetyltransferase in human lung cancer cells by the bis(ethyl)polyamine analogues. Cancer Res 1989;49:3829-33.

15. McCloskey DE, Coleman CS, Pegg AE. Properties and regulation of human spermidine/spermine N^1-acetyltransferase stably expressed in Chinese hamster ovary cells. J Biol Chem 1999;274:6175-82.

16. Chen Y, Kramer DL, Diegelman P, Vujcic S, Porter CW. Apoptotic signaling in polyamine analogue-treated SK-MEL-28 human melanoma cells. Cancer Res 2001;61:6437-44.

17. Porter CW, Ganis B, Libby PR, Bergeron RJ. Correlations between polyamine analogue-induced increases in spermidine/spermine N^1-acetyltransferase activity, polyamine pool depletion, and growth inhibition in human melanoma cell lines. Cancer Res 1991;51:3715-20.

18. Hahm HA, Ettinger DS, Bowling K, et al. Phase I study of N^1,N^{11}-diethylnorspermine in patients with non-small cell lung cancer. Clin Cancer Res 2002;8:684-90.

19. Creaven PJ, Perez R, Pendyala L, et al. Unusual central nervous system toxicity in a Phase I study of N^1,N^{11} diethylnorspermine in patients with advanced malignancy. Invest New Drugs 1997;15:227-34.

20. Vujcic S, Liang P, Diegelman P, Kramer DL, Porter CW. Genomic identification and biochemical characterization of the mammalian polyamine oxidase involved in polyamine back-conversion. Biochem J 2003;370:19-28.

21. Urdiales JL, Medina MA, Sanchez-Jimenez F. Polyamine metabolism revisited. Eur J Gastroenterol 2001;13:1015-9.

22. Vujcic S, Diegelman P, Bacchi CJ, Kramer DL, Porter CW. Identification and characterization of a novel flavin-containing spermine oxidase of mammalian cell origin. Biochem J 2002;367:665-75.

23. Wu T, Yankovskaya V, McIntire WS. Cloning, sequencing, and heterologous expression of the murine peroxisomal flavoprotein, N^1-acetylated polyamine oxidase. J Biol Chem 2003;278:20514-25.

24. Chen Y, Vujcic S, Liang P, Diegelman P, Kramer DL, Porter C. Genomic Identification and biochemical characterization of a second spermidine/spermine N^1-acetyltransferase. Biochem J 2003;373:661-7.

25. Xiao L, Celano P, Mank AR, et al. Structure of the human spermidine/spermine N^1-acetyltransferase gene (exon/intron gene organization and localization to Xp22.1). Biochem Biophys Res Commun 1992;187:1493-502.

26. Vujcic S, Halmekyto M, Diegelman P, et al. Effects of conditional overexpression of spermidine/spermine N^1-acetyltransferase on polyamine pool dynamics, cell growth, and sensitivity to polyamine analogs. J Biol Chem 2000;275:38319-28.

27. Chen Y, Kramer D, Jell J, Vujcic S, Porer CW. siRNA suppression of polyamine analogue-induced spermidine/spermine N^1-acetyltransferase. Mol Pharmacol 2003;64:1153-9.

28. Rubinstein LV, Shoemaker RH, Paull KD, et al. Comparison of *in vitro* anticancer drug screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. J Natl Cancer Inst 1990;82:1113-8.

29. Fogel-Petrovic M, Shappell NW, Bergeron RJ, Porter CW. Polyamine and polyamine analog regulation of spermidine/spermine N^1-acetyltransferase in MALME-3M human melanoma cells. J Biol Chem 1993;268:19118-25.

30. Fogel-Petrovic M, Vujcic S, Miller J, Porter CW. Differential post-transcriptional control of ornithine decarboxylase and spermidine-spermine N^1-acetyltransferase by polyamines. FEBS Lett 1996;391:89-94.

31. Fogel-Petrovic M, Vujcic S, Brown PJ, Haddox MK, Porter CW. Effects of polyamines, polyamine analogs, and inhibitors of protein synthesis on spermidine-spermine N^1-acetyltransferase gene expression. Biochemistry 1996;35:14436-44.

32. Smith PF, Booker B, Pendyala L, et al. Pharmacokinetic modeling of oxaliplatin with and without 5-FU and radiation. Proc AACR 2001;42:542.

33. Graham MA, Lockwood GF, Greenslade D, Brienza S, Bayssas M, Gamelin E. Clinical pharmacokinetics of oxaliplatin: a critical review [Review] [49 refs]. Clin Cancer Res 2000;6:1205-18.

34. Fogel-Petrovic M, Kramer DL, Vujcic S, et al. Structural basis for differential induction of spermidine/spermine N^1-acetyltransferase activity by novel spermine analogs. Mol Pharmacol 1997;52:69-74.

35. Seiler N. Functions of polyamine acetylation [Review] [114 refs]. Can J Physiol Pharmacol 1987;65:2024-35.

36. Maxwell P, Longley DB, Latif T, et al. Identification of 5-fluorouracil-inducible target genes using cDNA microarray profiling. Cancer Res 2003; 63:4602-6.

37. Shappell NW, Miller JT, Bergeron RJ, Porter CW. Differential effects of the spermine analog, N^1,N^{12}-bis(ethyl)-spermine, on polyamine metabolism and cell growth in human melanoma cell lines and melanocytes. Anticancer Res 1992;12:1083-9.

38. McCloskey DE, Pegg AE. Altered spermidine/spermine N^1-acetyltransferase activity as a mechanism of cellular resistance to bis(ethyl)polyamine analogues. J Biol Chem 2000;275:28708-14.

39. Kee K, Vujcic S, Kisiel N, Diegelman P, Kramer D, Porter CW. Activation of polyamine catabolism as a novel strategy for treating and/or preventing prostate cancer. Proc AACR 2003;44:1277.

40. Hawthorne TR, Kramer Austin J Jr. Synergism of the polyamine analog, N^1,N^{11}-bisethylnorspermine with *cis*-diaminedichloroplatinum (II) against murine neoplastic cell lines *in vitro* and *in vivo*. Cancer Lett 1996; 99:99-107.

41. Marverti G, Piccinnini G, Ghaironi S, Barbieri D, Quaglino D, Moruzzi MS. N^1,N^{12}-bis(ethyl)spermine effect on growth of *cis*-diamminedichloroplatinum(II)-sensitive and -resistant human ovarian-carcinoma cell lines. Int J Cancer 1998;78:33-40.

42. Paliwal J, Janumpalli G, Basu HS. The mechanism of polyamine analog-induced enhancement of cisplatin cytotoxicity in the U-251 MG human malignant glioma cell line. Cancer Chemother Pharmacol 1998;41:398-402.

43. Marverti G, Bettuzzi S, Astancolle S, Pinna C, Monti MG, Moruzzi MS. Differential induction of spermidine/spermine N^1-acetyltransferase activity in cisplatin-sensitive and -resistant ovarian cancer cells in response to N^1,N^{12}-bis(ethyl)spermine involves transcriptional and post-transcriptional regulation. Eur J Cancer 2001;37:281-9.