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ORIGINAL ARTICLE

Combination effects of platinum drugs and N^1, N^{11} diethylnorspermine on spermidine/spermine N^1-acetyltransferase, polyamines and growth inhibition in A2780 human ovarian carcinoma cells and their oxaliplatin and cisplatin-resistant variants

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Abstract
Purpose To understand the mechanisms behind platinum drug/DENSPM-induced inhibition of cancer cell growth, we compared the effects of oxaliplatin and cisplatin when combined with DENSPM on the induction of SSAT mRNA, activity, polyamines and cell growth in A2780 human ovarian carcinoma cells and their oxaliplatin- and cisplatin-resistant variants A2780/C10B and A2780/CP, respectively.
Methods Parental and Pt-resistant cells were treated with platinum agent alone, DENSPM alone or combination (10 μM each, 20 h). QRT–PCR, radioactive product measurement and HPLC were used for mRNA, activity and polyamine pools, respectively; drug interaction on cell growth was by SRB and isobologram analysis.
Results Both platinum agents induced SSAT mRNA in parental A2780 cells, but not in resistant cells. Platinum drug/DENSPM combinations produced high levels of SSAT activity in parental cells with significant depletion of spermine and spermidine, but not in resistant cells. Co-treatment with platinum agents increased the levels of DENSPM in all cell lines. Oxaliplatin/DENSPM combination was superior to cisplatin/DENSPM in the inhibition of cell growth in parental cells. No synergy was observed in the resistant cells.
Conclusions Increased DENSPM levels following co-treatment with Pt agents enhances the translation and stability of SSAT protein leading to polyamine pool depletion, facilitating more Pt–DNA adduct formation in parental cells. Oxaliplatin/DENSPM combination is superior to cisplatin/DENSPM in cell growth inhibition as DACH-Pt DNA adducts are cytotoxic even at relatively fewer numbers. Reduced platinum uptake in Pt-resistant cells contributes to reduced SSAT mRNA induction and absence of synergy when combined with DENSPM.

Keywords Oxaliplatin · Cisplatin · Platinum resistance · Polyamines · Diethylnorspermine

Abbreviations

N^1AcSpd	N^1acetylspermidine
BESPM	Bisethylspermine
DEHSPM	N^1, N^{14}-diethylhomospermine
DACH-Pt	Diaminocyclohexane platinum
DENSPM	N^1N^{11}-diethylnorspermine
5FU	5-Fluorouracil
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
Pt	Platinum
Put	Putrescine
QRT–PCR	Quantitative RT–PCR
ROS	Reactive oxygen species

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SAMDC	*S*-adenosylmethionine decarboxylase
SMO	Spermine oxidase
Spd	Spermidine
Spm	Spermine
SSAT	Spermidine/spermine N^1-acetyltransferase
SRB	Sulforhodamine-B

Introduction

Platinum (Pt) drugs cisplatin and oxaliplatin play a central role in cancer chemotherapy. The prototype drug cisplatin is used in ovarian, testicular, bladder, small cell lung and head and neck cancers among many others. Oxaliplatin while active in some of the above cancers is uniquely active in colon carcinoma when administered in combination with 5-fluorouracil and has been approved for clinical use for this indication. It is widely believed that Pt–DNA adduct formation is the key lesion for the activity of these agents preventing DNA replication and transcription and the resulting DNA damage itself inducing downstream signaling leading to apoptotic cell death [45]. However, all platinum drugs also bind to RNA and protein within the cell and these interactions may also play a role in their mechanism of action. Mechanisms of resistance to platinum agents are multifactorial including impaired drug accumulation, inactivation by thiols, enhanced DNA repair, replicative bypass, tolerance to damage and altered downstream signaling [17, 36, 37, 39].

Microarray studies on oxaliplatin- [4, 42] or cisplatin [42]-treated cells have suggested that platinum drugs can affect the expression of genes in many other pathways and identified spermidine/spermine N^1 acetyl transferase (SSAT) a key polyamine catabolic pathway enzyme as a prominently up-regulated gene. Other microarray studies have identified SSAT up-regulation by oxaliplatin in HCT-116 colon carcinoma cells [1, 4] and MCF-7 cells and by 5-fluorouracil (5-FU) in HCT-116 cells [1, 47] and MCF-7 cells [31]. Our own gene expression profiling studies of oxaliplatin- or cisplatin-treated A2780 ovarian carcinoma cells indicated that both drugs not only up-regulate SSAT gene expression but they also down-regulate the polyamine biosynthetic pathway enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) [42]. We and others have found that oxaliplatin up-regulated SSAT expression is not translated into protein and/or activity to any significant extent [1, 23, 24]; but when combined with the polyamine analog N^1,N^{11}diethylnorspermine (DENSPM), a synergistic induction of both SSAT mRNA [1, 23, 24], protein [1] and activity occurs [23, 24]. This is not surprising because both SSAT mRNA and protein have short half-lives and polyamine analogs have been shown to facilitate increased transcription and the stability of the transcript [7] as well as increase the translation and stability of the SSAT protein [7].

Polyamines, small cationic molecules which are essential for cell proliferation and survival, are a well-recognized therapeutic target. SSAT is the key enzyme in polyamine catabolism and its induction leads to inhibition of cell growth and apoptosis [9, 25]. Because polyamine levels and metabolic rates are higher in tumor than in normal cells [34], drugs have been designed to either up-regulate polyamine catabolism or down-regulate their biosynthesis. The clinically tested polyamine analog DENSPM targets the polyamine pathway by up-regulating catabolism *via* SSAT induction and decreasing their biosynthesis *via* down-regulation of the biosynthetic enzymes ODC and SAMDC [41]. DENSPM has been tested in Phase I and Phase II clinical trials [11, 19, 40, 46], but with no demonstrated single agent activity. A few studies, including our own, have shown that oxaliplatin and DENSPM combination synergistically up-regulates the expression of SSAT in A2780 [23] and HCT-116 cells [1, 10, 24] with concurrent decline in polyamine pools [10, 23, 24]. Others have shown that DENSPM combined with paclitaxel or 5-FU produces a synergistic induction of SSAT mRNA and activity in MDA-MB-231 cells [33]. The synergistic induction of SSAT protein and activity by oxaliplatin/DENSPM combinations in HCT-116 and other colon carcinoma cell lines and A2780 ovarian carcinoma cells result in synergistic cell kill [1, 24] or enhancement of oxaliplatin cytotoxicity [23]. While exhibiting lower levels of synergy in SSAT induction after oxaliplatin/DENSPM combination, the oxaliplatin-resistant HCT-116 cells showed an enhanced cell death following treatment with this drug [1]. In addition, siRNA-mediated down-regulation of SSAT resulted in decreased cell kill [1]. Cisplatin, DENSPM combinations have been shown to produce synergistic cell death in MDA-MB-231 breast cancer cells [33]. Mareverti et al., have shown that another polyamine analog N^1, N^{12}-bis(ethyl)spermine (BESPM) induced SSAT at much lower levels in cisplatin-resistant C13* ovarian carcinoma cells than the parental cells but the combinations of cisplatin and BESPM still produced synergistic cell kill [29]. All these lines of evidence suggest SSAT induction to be a potential therapeutic target in cancer chemotherapy.

While recent studies focused on combining the clinically relevant DENSPM with oxaliplatin, detailed studies of cisplatin combinations with DENSPM on polyamine pathway and growth are lacking in ovarian cancer cells. The aim of the present work has been to conduct a simultaneous comparison of the interactions of DENSPM with cisplatin and oxaliplatin under identical conditions at the level of SSAT mRNA, activity, polyamine pools and the effect of the combination on inhibition of cell growth in

A2780 ovarian carcinoma cells and their corresponding oxaliplatin and cisplatin-resistant variants A2780/C10B and A2780/CP, respectively. The mechanisms of resistance for both A2780/CP and A2780/C10B have been described previously and include decreased drug accumulation and increased glutathione for both cell lines and in addition increased repair of DNA damage in cisplatin-resistant A2780/CP [21, 22].

Materials and methods

Drugs

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

Cell culture

All cell lines were of human origin and *mycoplasma*-free. The ovarian carcinoma cell line A2780 and its cisplatin-resistant variant A2780/CP were a gift from Dr. Ozols (Fox Chase Cancer Center, Philadelphia, PA). The oxaliplatin-resistant A2780/C10B subline was derived in our laboratory as a single cell clone from the previously described stably oxaliplatin-resistant A2780/C10 cell line using limit-dilution procedure [22, 43]. Both A2780/CP and A2780/C10B variants are stably resistant when maintained in drug-free medium. All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Grand Island, NY) and 1% L-Glutamine (Invitrogen, Grand Island, NY).

Cell growth inhibition

Cells were plated in a 96-well plate (1×10^3 cells/well) on day 0 followed by exposure to oxaliplatin or cisplatin on day 1. Cells were exposed to drug concentrations ranging from 0.1 to 100 μM for 72 h, after which the cells were fixed and subjected to the sulforhodamine-B micro-culture colorimetric assay (SRB) [38]. Percent survival was determined as (OD_{570} (treated cells)/OD_{570} (untreated cells)) $\times$ 100.

Drug treatment conditions for measurement of SSAT gene expression, SSAT enzyme activity and polyamine pools

Cells were treated concurrently with either of the platinum agents (10 μM) and DENSPM (10 μM) for 20 h, washed thoroughly with PBS, and incubated for another 24 h in drug-free medium prior to harvesting the cells. Single agent treatments were performed under identical exposure and incubation conditions. The concentrations chosen for all the drugs are clinically achievable [18, 19, 26] and the drug exposure time reflects the half-life observed for oxaliplatin [18]. In addition, combining DENSPM with oxaliplatin under these conditions produced synergistic increases in SSAT mRNA and activity in A2780 cells [23] and in HCT-116 cells [24]. The same exposure time was chosen for cisplatin to provide a comparative characterization with oxaliplatin. The post-drug treatment incubation of 24 h in drug-free medium was chosen based on our previous studies [42] which showed that SSAT gene expression increases with time up to 24 h after the end of platinum drug exposure. Control cells were incubated identically in the absence of drug.

Real time quantitative RT-PCR

SSAT mRNA levels were measured by real-time quantitative RT-PCR (Taqman assay) using PE-ABI 7900 Sequence Detection System as described before [24]. The mRNA level is expressed relative to the endogenous standard (β-actin) measured concurrently from the same RNA extracts and the cDNA preparations. The drug untreated control is the calibrator in these assays and fold induction relative to the calibrator is presented.

SSAT activity measurements

SSAT activity assay was performed as described previously [35]. In brief, the reaction mixture contained [^{14}C] acetylCoA (60 mCi/mmol, NEN Radiochemicals, Waltham, MA) spermidine and cell extract in Tris HCl buffer, pH 7.5. The [^{14}C]acetylated spermidine product generated by the enzyme reaction is captured on discs followed by counting of radioactivity. Protein in the cell lysate was determined by the Bradford assay [5]. The activity reported is 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 HPLC with fluorescence detection as previously described [44]. Polyamine pools are expressed as pmol/mg protein.

Drug interaction on cell growth inhibition

The effect of two drug combination on cell growth was examined as described by Faessel et al. [13]. Exponentially growing A2780, A2780/C10B and A2780/CP cell lines

were seeded in 96-well microtiter plates in drug-free media and incubated for 24 h. Treatment with oxaliplatin or cisplatin, and the combination of each of the platinum drugs and DENSPM was then carried out in fixed binary ratio mixtures (1:4, 1:2, 1:1, 2:1, 4:1) of each drug at their IC_{50} concentrations in each of the cell lines. Each experiment consisted of 5 plates and each plate included 12 wells each for control, oxaliplatin, DENSPM and each of the 5 combinations of oxaliplatin and DENSPM in constant ratio with 12 dilutions. The serial dilutions were randomized as described earlier [12, 13]. Following 96 h of drug exposure, the cells were fixed, stained with sulforhodamine-B (SRB), washed thoroughly to remove the excess dye and absorbance read at 570 nm and inhibition of cell growth was determined. The data were de-randomized and analyzed. Experiments for each cell line were repeated 2–4 times, such that the data analyzed were obtained from 10 to 20 separate plates.

Modeling of the oxaliplatin/DENSPM interaction on cell growth inhibition

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 [16] 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.

$$E = B + \frac{(Econ - B)\left(\frac{C}{IC_{50}}\right)^{m}}{1 + \left(\frac{C}{IC_{50}}\right)^{m}}$$

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 WinNonlin Pro., Version 5.2 with nonlinear regression using the Gauss–Newton Minimization Method, with the estimation of the four parameters. The goodness-of-fit of the pharmacodynamic (PD) model to the cell inhibition data was assessed using a combination of the following criteria: the Akaike Information Criterion, sum of weighted squared residuals, percent coefficient of variation (%CV) of the estimated PD parameters, visual inspection of the predicted and observed concentration-effect profiles and residual (difference between observed and predicted) plots. A uniform weighting scheme was used for the analysis. Isobol graphs are then made from these data, at the IC_{10}, IC_{25}, IC_{50}, IC_{75} and IC_{90} levels, using SAS Version 8.2. Bowing of the isobols toward the Southwest corner of the graph indicates synergy; whereas bowing of the isobols toward the Northeast corner of the graph indicates antagonism; and when the isobols follow the diagonal additivity line, additivity is indicated.

Statistical analysis

Differences in cellular mRNA levels, enzyme activity and polyamine pools before and after drug treatments were evaluated using Student's t test (Systat Software Inc., Richmond, CA). P values ≤ 0.05 were considered significant.

Results

Cytotoxicity profiles of the platinum-resistant cells

The cytotoxicity profiles of oxaliplatin in A2780/C10B compared to the parental A2780 cells is shown in Fig. 1a. Compared to the parental A2780 cells, the A2780/C10B subline is 15-fold resistant to oxaliplatin; IC_{50} concentrations of A2780 and A2780/C10B are 0.2 and 3.0 μM, respectively. In Fig. 1b is shown the cytotoxicity profile for cisplatin in A2780 and the cisplatin-resistant variant A2780/CP. Compared to the parental cells, the A2780/CP cells are ~12 fold resistant to cisplatin; IC_{50} concentrations for cisplatin in A2780 and A2780/CP are 0.54 and 6.58 μM, respectively.

SSAT induction and polyamine pools

The SSAT mRNA, activity and polyamine pools were measured in the parental A2780 and the oxaliplatin-resistant A2780/C10B and the cisplatin-resistant A2780/CP cells. For these experiments, the cells were treated with each of the platinum agents or DENSPM (10 μM each) singly and in combination for 20 h followed by a 24-h incubation in drug-free medium. The rationale for choosing these conditions is presented in "Materials and methods". As seen in Fig. 2a, b, both platinum agents induced SSAT mRNA in A2780 cells, better than DENSPM in this cell line, oxaliplain producing a 61-fold increase and cisplatin a 42-fold increase relative to the no-drug controls. DENSPM treatment resulted in a small increase in SSAT mRNA (~16-fold) as we have noted also before for this cell line [23]. The differences between oxaliplatin and DENSPM are significant ($P = 0.028$). When combined with DENSPM, both oxaliplatin and cisplatin induced a significant further increase in SSAT mRNA in the parental A2780 cells; oxaliplatin/DENSPM combination produced a ~96-fold induction, while cisplatin/DENSPM produced a 69-fold induction. While this induction for the combination is significant for both platinum agents in comparison with

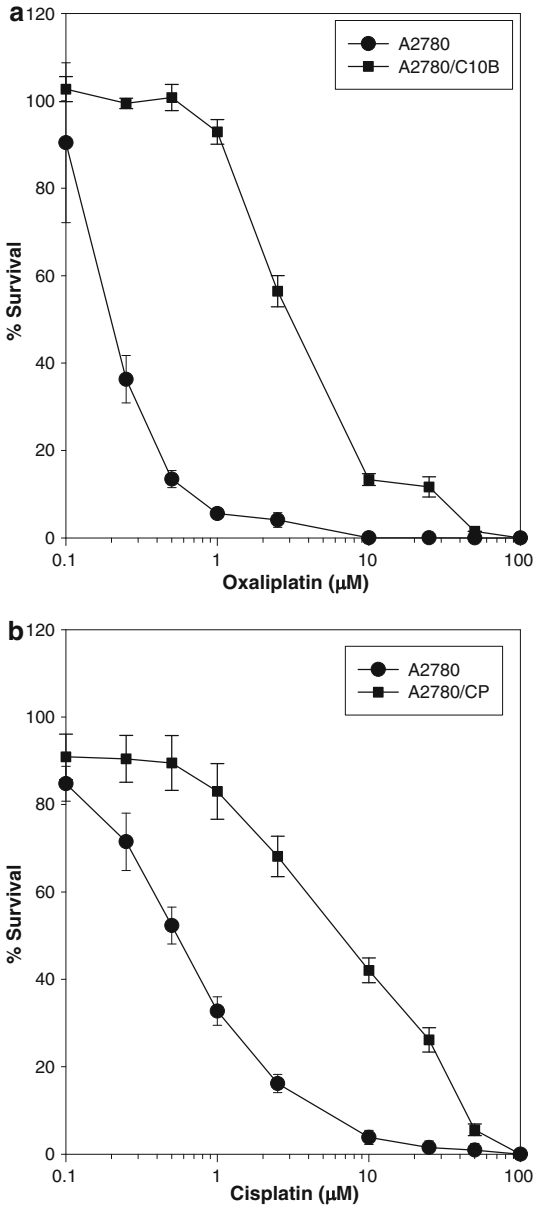


Fig. 1 **a** Comparative dose–response curves for oxaliplatin in A2780 ovarian carcinoma and the oxaliplain-resistant A2780/C10B cells following a 72-h continuous exposure to oxaliplatin. **b** Comparative dose–response curves for cisplatin in A2780 ovarian carcinoma cells and the cisplatin-resistant A2780/CP cells following a 72-h continuous exposure to cisplatin. Data represent mean ± SE where $n = 3$. The IC_{50} for oxaliplatin in A2780 and A2780/C10B are 0.2 and 3.0 μM, respectively. The IC_{50} for cisplatin in A2780 and A2780/CP are 0.54 and 6.58 μM, respectively

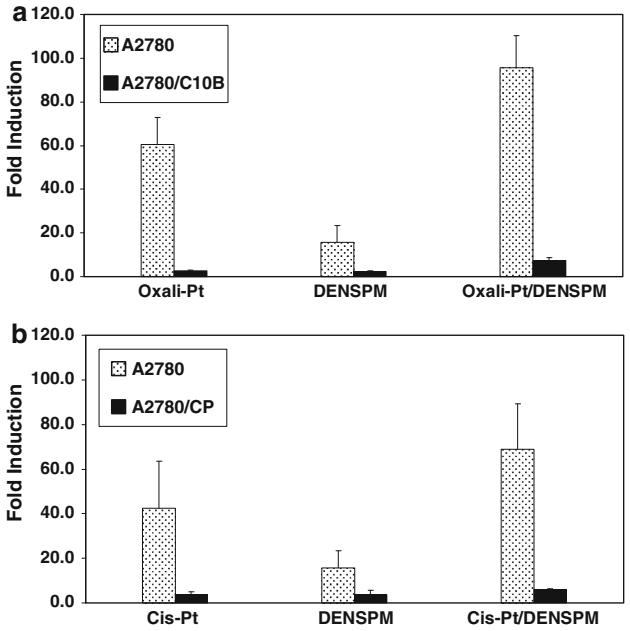


Fig. 2 **a** Effect of oxaliplatin alone, DENSPM alone or oxaliplatin/DENSPM combination on SSAT mRNA in A2780 and A2780/C10B cells. **b** Effect of cisplatin alone, DENSPM alone or cisplatin/DENSPM combination on SSAT mRNA in A2780 and A2780/CP cells. Cells were exposed to 10 μM platinum agent or 10 μM DENSPM singly, or in combination for 20 h followed by another 24-h incubation in drug-free medium prior to SSAT mRNA measurements. Data shown are fold induction relative to drug untreated controls. Data represent mean ± SE where $n = 2$–5 separate cell pellets in up to 3 separate experiments. In **a**, the fold-induction in A2780 after oxaliplatin, DENSPM and oxaliplatin/DENSPM are 60.6 ± 12.3, 15.8 ± 7.8 and 95.5 ± 15, respectively; the fold induction in A2780/C10B cells after the same treatments are 2.8 ± 0.1, 2.3 ± 0.4, and 7.4 ± 1.3, respectively. In **b**, the fold-induction in A2780 cells after cisplatin, DENSPM and cisplatin/DENSPM are 42.3 ± 1.1, 15.8 ± 7.8 and 69.0 ± 20.2, respectively; the fold induction in A2780/CP cells after the same treatments are 3.5 ± 1.3, 3.7 ± 1.9 and 5.9 ± 0.6, respectively

DENSPM ($P < 0.05$), it was not significant in comparison with platinum agent alone. Unlike the parental cells, the SSAT mRNA induction is significantly curtailed both in the oxaliplatin-resistant A2780/C10B cells and in the cisplatin-resistant A2780/CP cells. In comparison with the single agents, the drug combination produced a slight but not significant increase in SSAT mRNA in both resistant cell lines. The differences in mRNA induction between the parental and the resistant cells with single agent treatments as well as after combining the platinum agent with DENSPM are significant ($P < 0.05$). From these data, it is evident that acquired resistance to either platinum drug hinders the induction of SSAT mRNA both after single agent platinum treatment and in combination with DENSPM.

Although both platinum agents produced a significant increase in SSAT mRNA in A2780 cells it did not translate into a proportional increase in SSAT activity (Fig. 3a, b). Thus, oxaliplatin treatment produced a ∼4-fold increase in SSAT activity and a 2-fold increase after cisplatin relative to the corresponding no-drug controls in A2780 cells ($P < 0.01$ for both). A highly synergistic increase in SSAT activity occurred in these cells when each of the platinum agents was combined with DENSPM ($P < 0.001$), far

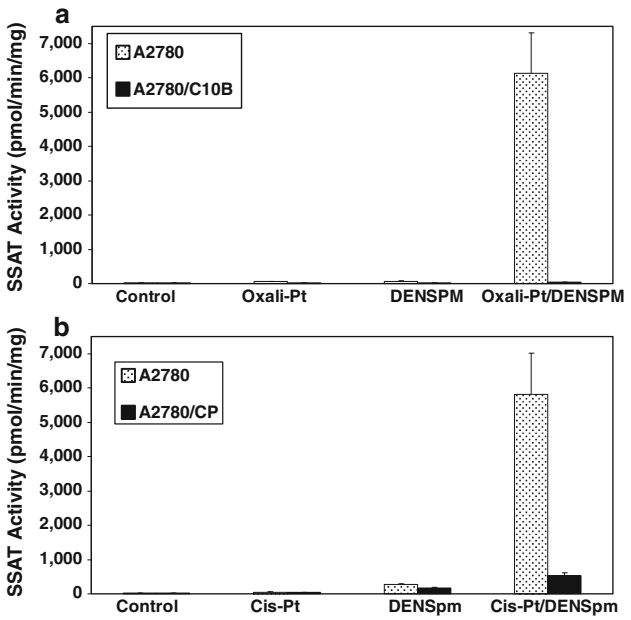


Fig. 3 **a** Effect of oxaliplatin alone, DENSPM alone or oxaliplatin/DENSPM combination on SSAT activity in A2780 and A2780/C10B cells. **b** Effect of cisplatin alone, DENSPM alone or cisplatin/DENSPM combination on SSAT activity in A2780 and A2780/C10B cells. Data shown are SSAT activity in pmol/min/mg protein. Data represent mean ± SE where $n = 3$–9 separate cell pellets in up to 3 separate experiments. In **a**, SSAT activity in A2780 for control (no-drug treatment) cells, and in cells treated with oxaliplatin, DENSPM and oxaliplatin/DENSPM were 15.0 ± 1.1, 57.3 ± 11.7, 53.9 ± 28.7 and 6130.2 ± 1182.0 pmol/min/mg protein, respectively; the same for A2780/C10B cells were 11.0 ± 3.0; 24.7 ± 3.8, 25.7 ± 1.8; 46.7 ± 0.9 pmol/min/mg protein, respectively. In **b**, SSAT activity in A2780 for control cells and in cells treated with cisplatin, DENSPM and cisplatin/DENSPM were 23.7 ± 1.7, 52.6 ± 12.5, 272.2 ± 30.1 and 5807 ± 1218 pmol/min/mg protein, respectively; the same for A2780/CP cells were 20.9 ± 5.5, 33.4 ± 8.1, 165.2 ± 18.7, 533 ± 76.9 pmol/min/mg protein, respectively

superior than any of the single agents. Under identical conditions of treatment oxaliplatin/DENSPM combination produced an average 400-fold increase in SSAT activity and cisplatin/DENSPM combination an average 245-fold increase; the differences between the two platinum agents were not statistically significant. Unlike the parental cells, the induction of SSAT activity with oxaliplatin/DENSPM is completely curtailed in the oxaliplatin-resistant A2780/C10B cells showing ~1% activity relative to the parental A2780 cells. The induction of SSAT activity observed in A2780/CP cells was <10% of that seen in A2780 cells. The differences between parental and resistant cells in inducing SSAT activity are highly significant for both oxaliplatin and cisplatin when combined with DENSPM ($P = 0.016$ to <0.001).

Polyamine pools were measured after treatment with oxaliplatin alone, DENSPM alone or a combination of the two under identical conditions as for SSAT mRNA and activity in the parental A2780 (Fig. 4a) and A2780/C10B (Fig. 4b) cells. Polyamine pools were also measured after treatment with cisplatin alone, DENSPM alone or a combination of cisplatin and DENSPM in A2780 (Fig. 4c) and the cisplatin-resistant A2780/CP (Fig. 4d) cells. Changes in putrescine (PUT), spermidine (SPD) and spermine (SPM) levels and that of N^1acetylspermidine (N^1AcSpd) levels under each of the treatment conditions relative to the untreated control are shown in Fig. 4a–d. DENSPM levels in cells were also measured. As evident from the data in both parental and platinum-resistant cells (A2780/C10B and A2780/CP), putrescine levels showed a tendency to increase with the drug treatment especially when the platinum drug and DENSPM were combined, while spermidine and spermine levels declined with the combination in all cells. The increases in putrescine, after oxaliplatin alone or when combined with DENSPM, were significant in both A2780 and A2780/C10B cells ($P < 0.05$ for all). The increases in putrescine after cisplatin alone or when combined with DENSPM, were significant only in A2780/CP cells ($P < 0.05$). As seen in Fig. 4a–d, the declines in spermidine and spermine pools produced by the platinum agent/DENSPM combinations in oxaliplatin-resistant A2780/C10B cells and the cisplatin-resistant A2780/CP cells are significantly lower than those observed in parental A2780 cells. The oxaliplatin/DENSPM combination produced 83 and 80% decline in spermidine and spermine, respectively in A2780 cells (Fig. 4a); similarly, the cisplatin/DENSPM combination produced 84% decline in spermidine but only 70% decline in spermine in parental cells (Fig. 4c), all declines being highly statistically significant for both platinum agents ($P < 0.001$). In A2780/C10B cells, the oxaliplatin/DENSPM combination produced a 30% decline in spermidine and 74% decline in spermine. The cisplatin/DENSPM treatment produced a decline of only 49% in spermidine and 47% of spermine in A2780/CP cells. Putrescine levels were found to be relatively higher in the cisplatin-resistant A2780/CP cells compared to the parental A2780 or A2780/C10B cells. The significance of this is unclear at this time.

A significant point of interest is that while spermidine and spermine levels declined in A2780 cells after DENSPM combinations with both platinum agents, the N^1acetylspermidine (N^1AcSpd) levels were measurable only after oxaliplatin and oxaliplatin/DENSPM combinations. The oxaliplatin/DENSPM combination produced significantly higher levels of N^1AcSpd compared to oxaliplatin alone in these cells ($P < 0.001$). There were relatively small but measurable levels of N^1AcSpd also in A2780/C10B but only in oxaliplatin/DESNPM-treated cells. Normally found in low or below detectable levels in un-induced cells, accumulation of intracellular acetylated polyamines is the best indicator of SSAT induction.

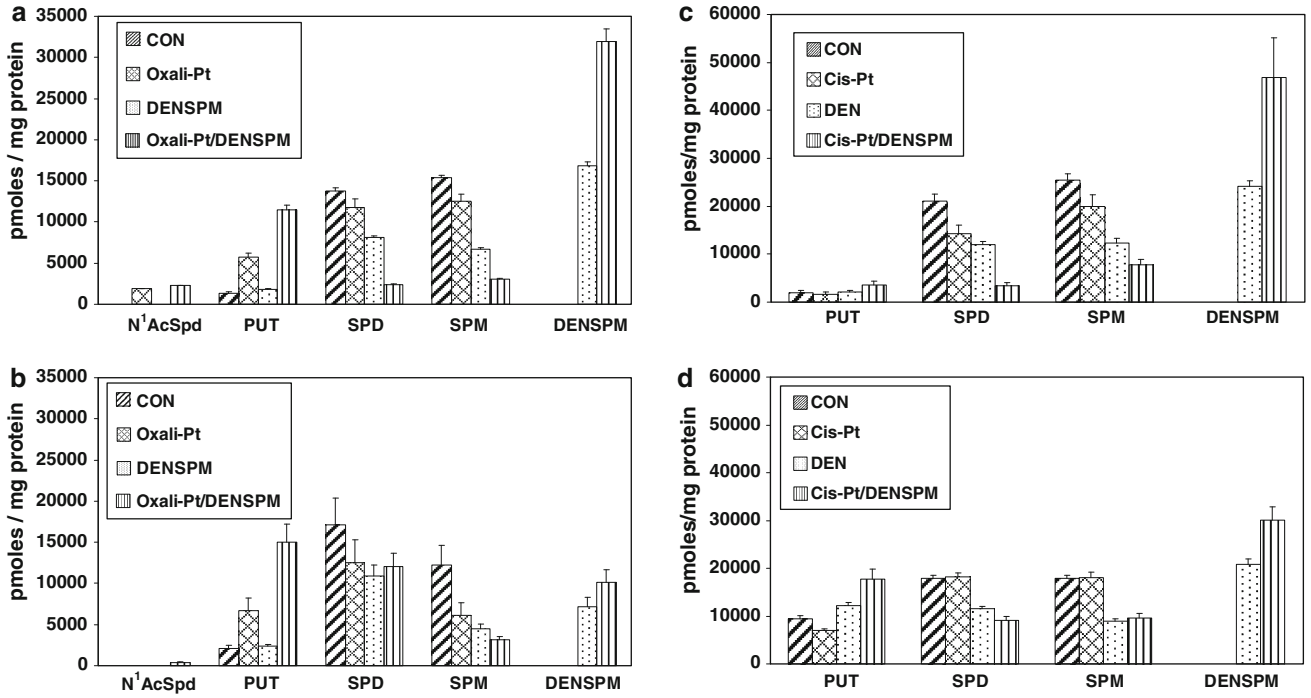


Fig. 4 Effect of oxaliplatin alone, DENSPM alone or oxaliplatin/DENSPM combination on polyamine pools in A2780 (**a**) and A2780/C10B (**b**) cells and cisplatin alone, DENSPM alone or cisplatin/DENSPM on polyamine pools in A2780 (**c**) and A2780/CP (**d**) cells. Data shown are pmoles/mg protein. Note that N^1 acetyl spermidine was detected only in oxaliplatin/DENSPM combinations. DENSPM levels in cells when administered alone or in combination with the platinum agent is also presented for all the cell lines. Note that DENSPM levels are higher when administered with the platinum agent. Data represent mean ± SE where $n = 3$–9 separate cell pellets in up to 3 separate experiments

The polyamine analog DENSPM was also measured in these experiments. In both parental and drug-resistant cells, under identical conditions, the DENSPM levels were higher in all cell lines when the treatment was in combination with the platinum agent. An approximate 200% increase was seen in DENSPM levels in A2780 cells when administered with either oxaliplatin or cisplatin ($P < 0.001$ for both). An approximate 150% increase in DENSPM was seen in A2780/CP cells after cisplatin/DENSPM ($P = 0.003$) and 140% increase in A2780/C10B cells after oxaliplatin/DENSPM, the later not statistically significant.

Cytotoxicity interactions between platinum agent and DENSPM

N^1acetylspermidine was detected only after oxaliplatin/DENSPM combination and not in cisplatin/DENSPM combinations. Likewise, the degree of SSAT activity induction by platinum agent/DENSPM combination differed markedly between sensitive and resistant cell lines. Therefore, we performed drug interaction analysis on cell growth using a wide range of concentrations and combination treatments of each of the platinum agents and DENSPM in A2780, A2780/C10B and A2780/CP cells. We hypothesized that SSAT induction was a determinant of the cytotoxic response to the drug combination. At all concentrations and ratios of oxaliplatin/DENSPM combination, the two drugs synergized to produce increased growth inhibition in A2780 parental cells (Fig. 5a). In contrast to combination with oxaliplatin, DENSPM combinations with cisplatin in A2780 cells were observed to display limited synergy in producing cell growth inhibition at low drug concentrations, which was reduced as the drug concentrations increased; a shift toward antagonism was evident at high concentrations (Fig. 5b). As opposed to the parental A2780 cells in A2780/C10B cells, the drug combination produced an antagonistic response and only a limited synergy at the highest doses and only in certain ratios (Fig. 5c). Cisplatin/DENSPM exhibited an antagonistic response at all concentrations at majority of the drug ratios in A2780/CP cells (Fig. 5d).

Discussion

The polyamine analog DENSPM is a proven inducer of SSAT in many cell types and has also been shown to down-regulate the key biosynthetic pathway enzymes ODC and SAMDC. Induction of SSAT activity and depletion of polyamine pools especially that of spermidine have been

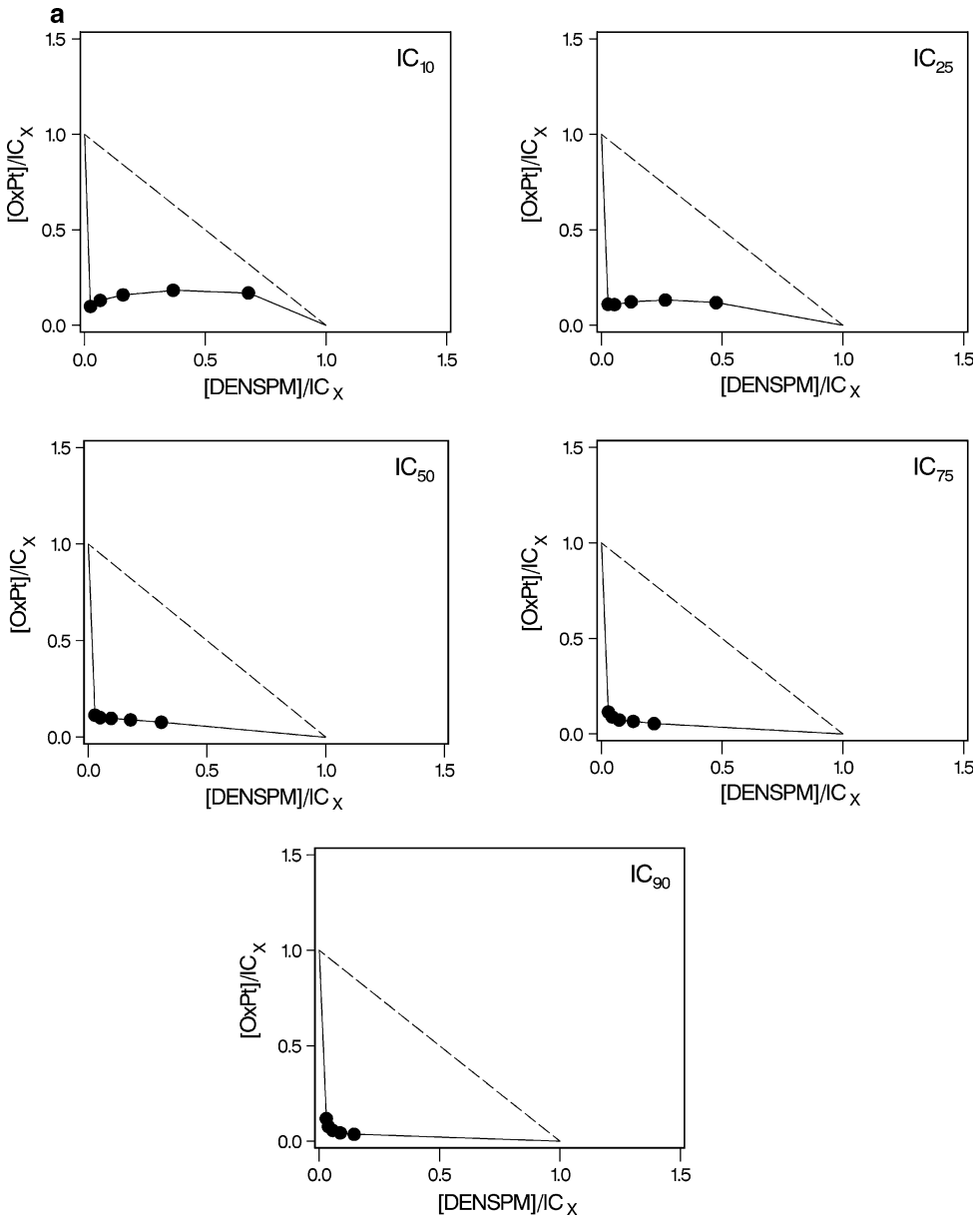


Fig. 5 **a** Isobolograms of oxaliplatin and DENSPM in combination treatment of A2780 cells. **b** Isobolograms of cisplatin and DENSPM in combination treatment of A2780 cells. **c** Isobolograms of oxaliplatin and DENSPM in combination treatment of A2780/C10B cells. **d** Isobolograms of cisplatin and DENSPM in combination treatment of A2780/CP cells. In the axis legend, '×' refers to the IC concentrations for each drug. The diagonal *dashed line* represents additivity and data below this line implies synergism and data above represent antagonism

shown to be related to inhibition of cancer cell growth [8, 27, 35, 44]. Therefore, this agent has great potential as a means of targeting the polyamine pathway as a mechanism to kill cancer cells and has accordingly been tested clinically [11, 19, 40, 46]. While this drug showed no single agent activity in the clinic, combination studies have yet to be reported. Several in vitro studies of synergistic cell growth inhibition of DENSPM combinations with various chemotherapeutic agents such as oxaliplatin [1, 24] and 5-fluorouracil [1] in colon carcinoma cell model, paclitaxel [33] and to a limited extent of cisplatin [33] in breast cancer cell models have been reported. Cisplatin is routinely used in ovarian cancer therapy and oxaliplatin has activity in this indication. Due to the importance of platinum agents in the treatment of this malignancy, it is imperative that we gain a better understanding of platinum agent/DENSPM combinations in ovarian cancer models and how this may eventually be brought to the clinical setting to improve treatment. While we noted from our earlier microarray studies that both platinum drugs impact on the polyamine pathway in A2780 ovarian carcinoma cells [42] and that oxaliplatin/DENSPM combinations synergize to produce high SSAT activity and lowered polyamines with enhancement of oxaliplatin cytotoxicity [23], a comparison of oxaliplatin and cisplatin with DENSPM combinations in the context of SSAT activity, polyamine pools and inhibition of cell growth has not been performed. To our knowledge, there were also no published reports on how cisplatin or oxaliplatin resistance may affect or modulate SSAT activity, polyamine pools and cell

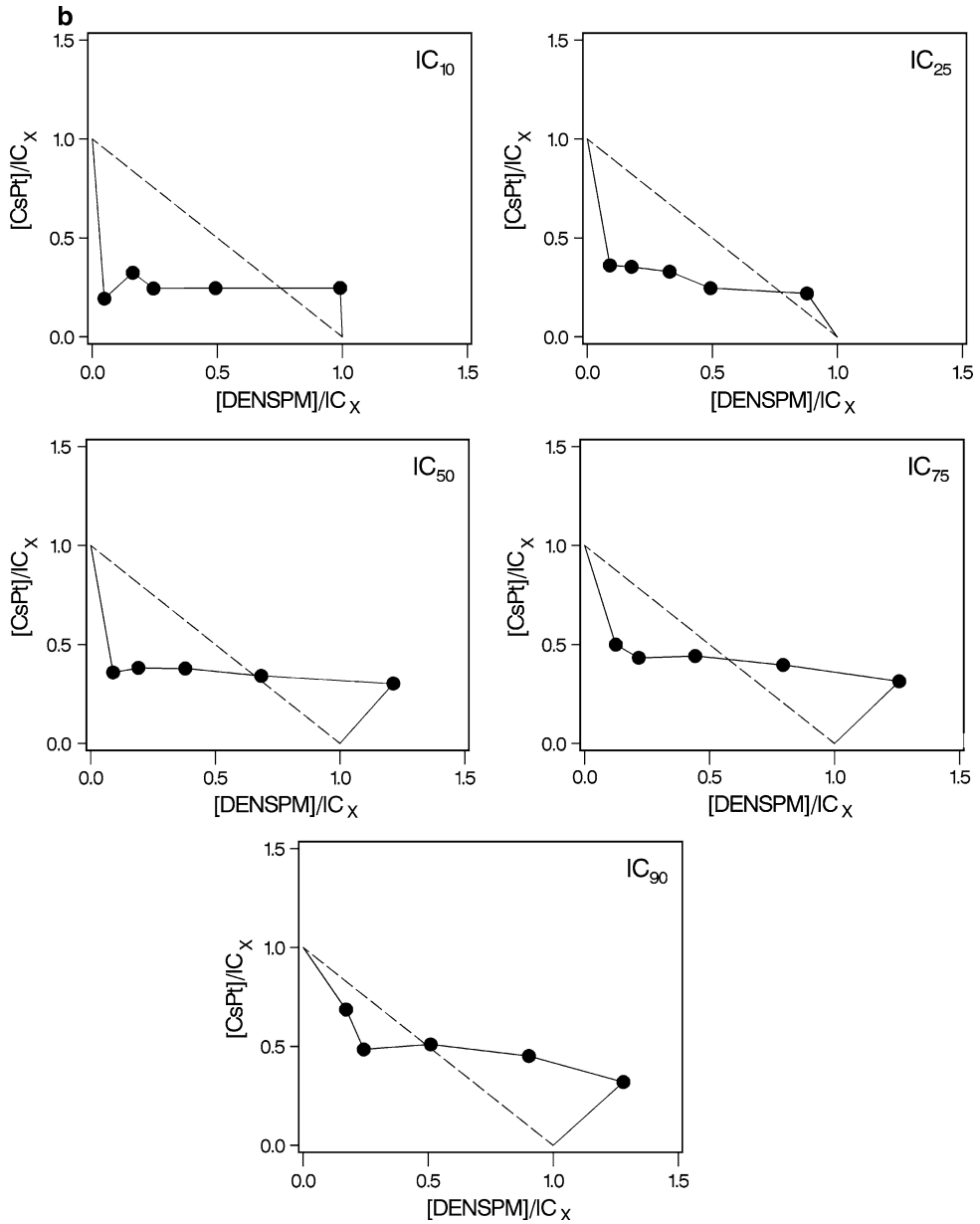
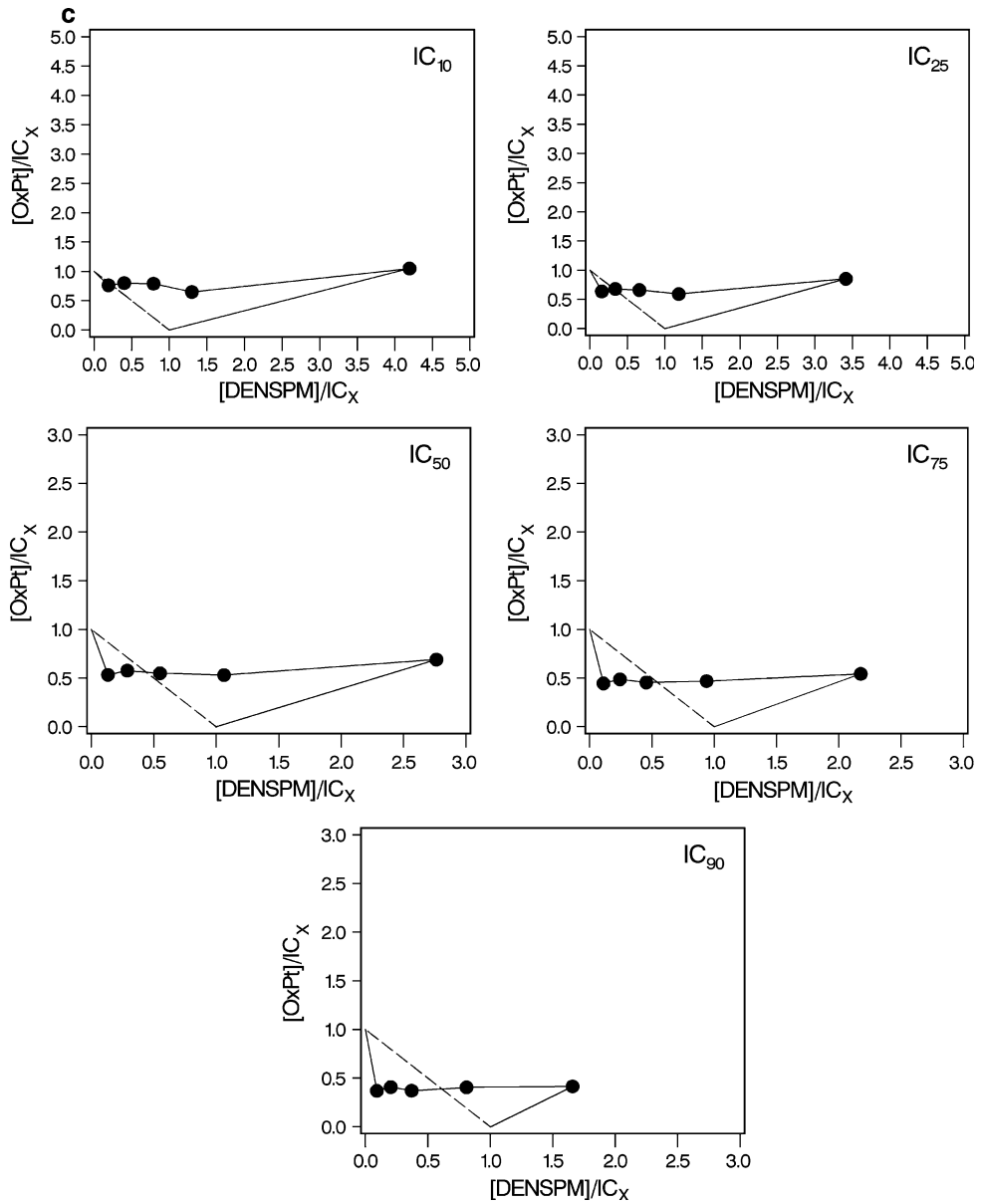


Fig. 5 continued

growth when platinum agents are combined with DENSPM in comparison with the parental cells in ovarian carcinoma cells.

We show here for the first time that when tested concurrently at a variety of concentrations and combinations with DENSPM, oxaliplatin synergizes with DENSPM in inhibiting cell growth better than cisplatin in this ovarian cancer cell model. It is intriguing why this is the case, as both agents produced high levels of SSAT activity and polyamine pool depletion when combined with DENSPM. It should, however, be emphasized that N^1acetylspermidine considered a good indicator of SSAT activity was detectable only in oxaliplatin/DENSPM combinations. Further, the measured fold increases in SSAT activity levels were higher and both spermine and spermidine were depleted highly in oxaliplatin/DENSPM combinations. In the current study, we observed that platinum agent/DENSPM combinations not only induced highly significant depletion of spermidine and spermine levels, but also a significant increase in putrescine levels. Whether this is due to back conversion of acetylated spermidine or increase in ornithine decarboxylase activity is unclear at this time.

We previously demonstrated that both oxaliplatin and cisplatin up-regulate spermine oxidase (SMO) and polyamine oxidase (PAO) in a concentration-dependent fashion



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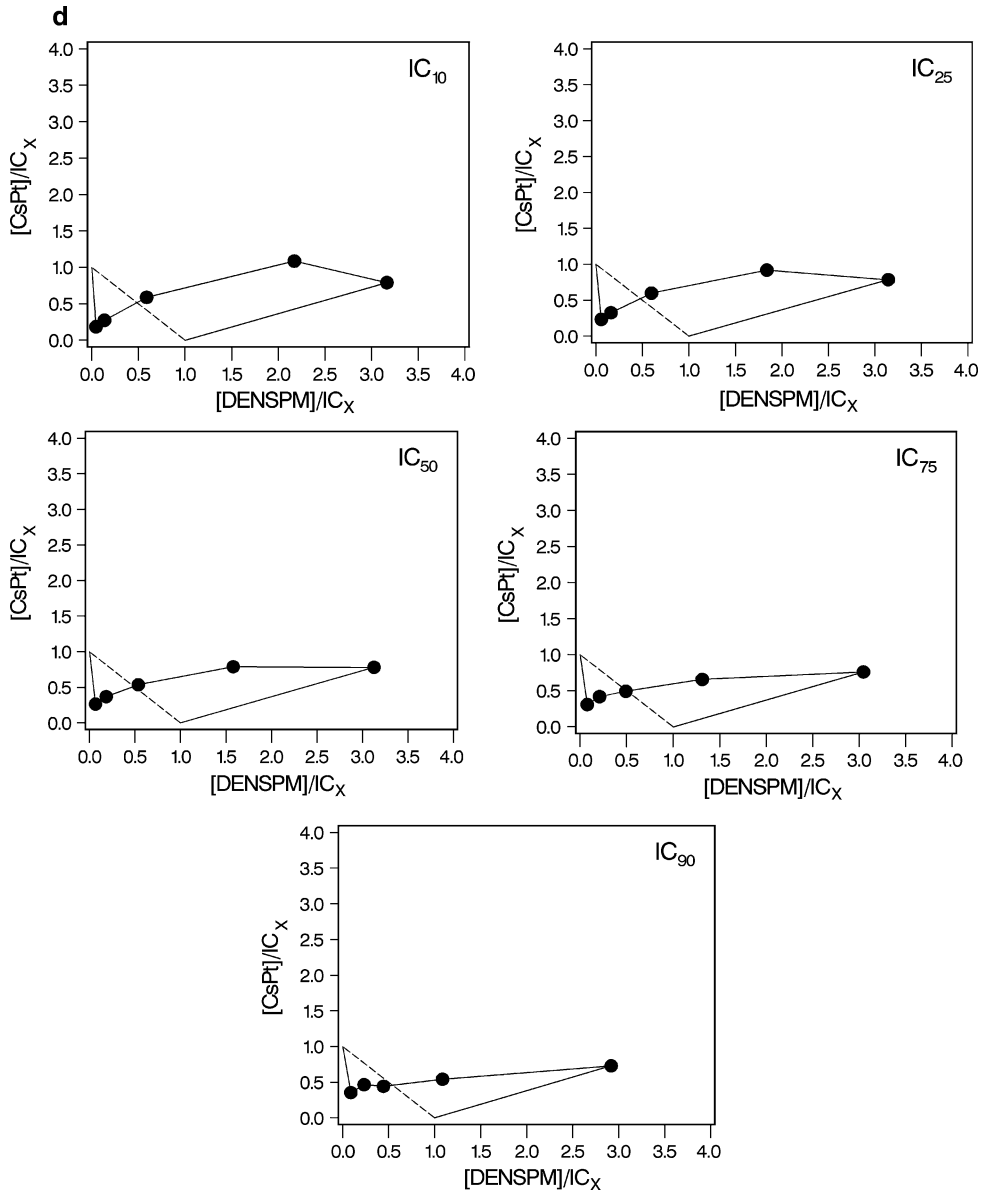
in A2780 cells [23]. Synergy between oxaliplatin and DENSPM was apparent for SMO up-regulation but not for PAO in A2780 cells [23]. We also demonstrated that oxaliplatin/DENSPM induced both mRNA and higher protein levels for SMO in human colon carcinoma HCT-116 cells [24]. Spermine oxidase (SMO) oxidizes spermine directly to spermidine; PAO preferentially oxidizes N^1acetylated polyamines to convert acetylated spermine and spermidine to spermidine and putrescine, respectively [6]. Oxidation of spermine to spermidine by SMO produces reactive oxygen species (ROS) down-stream to H_2O_2 generated as a reaction product [6]. Thus, one cannot rule out the possibility that additional factors such as increased production of highly cytotoxic ROS through SMO reaction may have contributed to the increased cytotoxicity of oxaliplatin/DENSPM.

Previous studies have shown synergism between oxaliplatin/DENSPM combinations on cell death in HCT-116 colon carcinoma cells resistant to oxaliplatin [1] and that for cisplatin and another polyamine analog BESPM in 2008 ovarian carcinoma cells resistant to cisplatin [29]. The detailed studies presented here exploring a variety of concentrations and ratios of the two drugs indicate that resistance to either oxaliplatin or cisplatin curtails the induction of SSAT, polyamine pool depletion and cell growth inhibition in A2780 cells. Thus, studies presented



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here comparing the parental with both the oxaliplatin-resistant and cisplatin-resistant cells indicate that the SSAT induction and polyamine pool depletion play a significant role in cell growth inhibition.

While the mechanisms of the platinum drug-polyamine interactions are not completely understood, based on the mechanism of action of polyamine analogs with respect to SSAT mRNA and protein [7] it seems likely that the synergistic increases in SSAT activity in the parental A2780 cells in our studies may likely be due to the enhanced stability and translation of platinum drug-induced SSAT mRNA and protein by DENSPM. SSAT activity is post-transcriptionally regulated such that SSAT mRNA requires a translational trigger by addition of a natural polyamine or analog [14, 15]. The evidence presented here indicates that cells with acquired resistance to platinum agents are deficient in their ability to up-regulate SSAT gene expression. It is important to note that lack of SSAT induction in the resistant cells in platinum agent/DENSPM combinations is not due to a DENSPM uptake deficiency. In fact, co-treatment with either of the platinum agents consistently increased the DENSPM levels in all parental and resistant cells under these conditions. This effect of the co-treatment with platinum drugs on DENSPM levels in cells has not been reported previously and appears to be a potentially significant contributing factor in the observed synergy in induction of SSAT activity in A2780 cells.

Whether increased DENSPM levels are a reflection of an increased uptake of DENSPM or decreased metabolism is unclear at this time as the experimental conditions consisted of 24 h incubation in drug-free medium following the 20-h combination treatment. DENSPM is known to be metabolized extensively in cells [2, 3]. Our previous studies showed that both the oxaliplatin-resistant A2780/C10B cells and the cisplatin-resistant A2780/CP cells are deficient in platinum uptake by ~50% relative to the parental cells under very similar conditions of testing as we described before [21, 22]. It is conceivable that the reduced platinum levels in the resistant cells have affected the SSAT up-regulation which appears to be the key determinant in platinum drug-DENSPM combination induced synergy at the SSAT activity level. We have previously reported the concentration-dependent up-regulation of SSAT gene expression for both oxaliplatin and cisplatin in A2780 ovarian carcinoma cells [23].

A synergistic interaction of cisplatin and DENSPM has been reported on growth inhibition in murine cell lines L-1210 leukemia and B16F1 melanoma both in vitro and in vivo [20]. Another study reported that pretreatment of cells with certain polyamine analogs (DEHSPM and DENSPM) increased the cytotoxicity of cisplatin in U-251 MG human malignant glioblastoma cells [32]. This later study suggested that pretreatment of cells with polyamine analogs increased the cisplatin DNA-adduct formation in the linker regions of DNA. Although synergy was noted in these earlier studies the rationale for combining the drugs was not explored with respect to SSAT induction and polyamine pool depletion in these studies. Since polyamines are cationic molecules that interact with the DNA and provide stability [41] it is highly likely that when polyamines are depleted there may be more interaction between platinum agents and DNA. Previous studies from our laboratory [21] and others have established that oxaliplatin is more potent than cisplatin [37]. Lower intracellular Pt-concentrations and fewer DNA-Pt adducts are sufficient for oxaliplatin-mediated cytotoxicity compared to cisplatin [21, 37]. It is possible that the observed superiority of oxaliplatin-DENSPM combination in cell growth inhibition in the present study may be related to potency of DACH Pt–DNA adducts relative to CisPt–DNA adduct in the cellular environment after polyamine pool depletion.

Marverti et al., reported that BESPM induces significantly lower levels of SSAT activity in C13* cells relative to sensitive 2008 cells due to decreased SSAT enzyme synthesis and high turnover in resistant cells [28]. Their work further suggested that during selection for cisplatin resistance a labile repressor protein was produced that regulates SSAT gene expression [30]. Using protein synthesis inhibitors, they observed enhanced transcription and stabilization of SSAT mRNA, as well as increased SSAT activity following BESPM treatment of C13* cells [30]. While their focus has been the polyamine analog-induced SSAT activity in cisplatin-resistant cells, it is possible that the highly curtailed SSAT gene expression in response to the corresponding platinum drugs in oxaliplatin and cisplatin-resistant cells in our study may be mediated through similar mechanisms, in addition to the effect of reduced platinum levels.

In conclusion, the studies presented here indicate that the synergy displayed by platinum drug/DENSPM combinations in inhibition of cell growth is a blend of a multitude of events. First, the platinum agents by themselves are potent inducers of SSAT mRNA in parental cells. Co-treatment with platinum agents increases the cellular levels of DENSPM which in turn facilitate increased translation and stability of SSAT protein and depletion of polyamine pools. In the cellular environment with decreased polyamine pools, Pt–DNA adducts may be more frequent. Fewer DACH Pt–DNA adducts being more cytotoxic than cisplatin–DNA adducts, oxaliplatin/DENSPM combination translates into better synergy in cell growth inhibition than cisplatin/DENSPM combination. The curtailed induction of SSAT gene expression in platinum resistance may be related at least partly to the defective platinum uptake in resistant cells. Further studies to define the molecular mechanisms behind platinum drug/DENSPM synergy will pave the way for realization of the full translational potential of these drugs.

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