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Reversal of doxorubicin, etoposide, vinblastine, and taxol resistance in multidrug resistant human sarcoma cells by a polymer of spermine

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Abstract We have previously descibed the synthesis of a cytotoxic polymeric conjugate of spermine (Poly-SPM) which is able to inhibit the transport of polyamines (spermine, spermidine, and putrescine) into normal and malignant cells. Recent studies examining the toxicity of Poly-SPM in parental and multidrug resistant (MDR) cancer cells have revealed a cross-resistance in the MDR variant Dx5 to the toxic effects of the conjugate in the MDR-positive cells. There were also differences in spermine and putrescine uptake rates between parental and MDR-positive cells with the MDR-positive cells having a lower V_{max} and a higher K_m. The ability of this Poly-SPM to reverse MDR was examined in MDR variants (Dx5 cells) of the human sarcoma cell line MES-SA. The cells express high levels of the mdr1 gene product, P-glycoprotein, and are 25- to 60-fold resistant to doxorubicin (DOX), etoposide (VP-16), vinblastine (VBL), and taxol (TAX). Cytotoxicity was measured by the MTT [3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Poly-SPM (50 μM) lowered the drug concentration IC_{50} values in the Dx5 cells by 37-fold with VBL, 42-fold with DOX, 29-fold with VP-16, and 25-fold with TAX when compared to the control IC_{50} values without Poly-SPM. This reversal of resistance was concentration dependent, decreasing 17-fold with DOX, 6.1-fold with VBL, 19-fold with VP-16, and 5-fold with TAX when 25 μM Poly-SPM was used. No modulation was observed in the parental cell line MES-SA, which does not express the mdr1 gene. Poly-SPM had no influence on the IC_{50} of non-MDR chemotherapeutic agents such as cisplatin. The modulation studies correlated with the ability of Poly-SPM to reverse the cellular accumulation defect of [^{3}H]-VBL and [^{3}H]-TAX in the Dx5 but not MES-SA cells. Pretreatment of the Dx5 cells with α-difluoromethylornithine (DFMO at 2 and 5 μM) for 24 h increased the function of the MDR transporter to further decrease the cellular accumulation of VBL and TAX when compared to untreated cells. DFMO pretreatment is known to upregulate the polyamine transporter(s). These findings show that, in addition to inhibiting polyamine transport, Poly-SPM reverses MDR in Dx5 cells, suggesting a potential relationship between the polyamine influx transporter and the MDR efflux pump. This potential functional link between the polyamine influx transporter(s) and the MDR efflux transporter (P-glycoprotein) offers a novel approach to inhibiting this form of drug resistance.

Key words Multidrug resistance · Polyamines · Difluoromethylornithine (DFMO)

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Introduction

Polyamines are a family of low molecular weight organic cations which are essential for cell growth and differentiation [1, 2]. All rapidly growing cells (including cancer cells), require a high quantity of polyamines to sustain proliferation [3–5]. Cellular polyamine contents appear to be regulated by both *de novo*

synthesis, governed primarily by ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC), and transmembrane transport [6, 7]. These observations have led many investigators to focus on targeting polyamine biosynthesis as a means of inhibiting cancer cell proliferation [7–9]. Several polyamine inhibitors and analogs have been developed which have antineoplastic activity and these are currently being tested in clinical trials [10–14]. One of the most studied inhibitors of polyamine biosynthesis is α-difluoromethylornithine (DFMO). While DFMO is a very potent ODC inhibitor, it affords relatively modest overall cellular polyamine depletion and minimal suppression of tumor cell proliferation. Though there are probably multiple reasons for the lack of effect of DFMO in these cells, it appears that an important compensatory mechanism is related to the induction of transmembrane polyamine transport, which can restore cell polyamine content and revitalize polyamine-dependent cellular functions [15–16]. Studies in tissue culture consistently show that DFMO causes large increases in transmembrane polyamine uptake [17]. Despite these intriguing observations, the utility of polyamine uptake pathways as targets for antineoplastic therapy has not been fully explored.

The development of drug resistance in human tumors is a major obstacle in the curative potential of cancer chemotherapeutics. One of these mechanisms of drug resistance is multidrug resistance (MDR). The typical MDR phenotype includes cross-resistance to anthracyclines, *Vinca* alkaloids, podophyllotoxins, taxanes, and other cytotoxic compounds, with increased expression of a membrane protein termed P-glycoprotein (P-gp). P-gp functions as an efflux pump, which is found on the surface membrane of resistant cells, and serves to transport antineoplastic agents out of the cancer cell in a manner similar to the polyamine influx transporters which carry polyamines into cancer cells [18, 19]. Reversal or modulation of MDR has been achieved using a wide variety of pharmacological agents including calcium channel blockers (e.g., verapamil, nifedipine, and diltiazem), cyclosporins (e.g., cyclosporin-A and PSC-833), cardiovascular drugs (e.g., dipyridamole and quinidine), steroid analogs (e.g., tamoxifen and progesterone), antibiotics (e.g., cefoperazone and erythromycin), calmodulin inhibitors (e.g., fluphenazine and trifluoperazine), and antimalarials (e.g., quinacrine and quinine). The mechanism by which most of these agents reverse MDR is by competitively inhibiting the binding of the chemotherapeutic agents to P-gp and is unrelated to their primary pharmacological mechanisms [18–22].

We have recently synthesized a polymeric conjugate of spermine (Poly-SPM) which is capable of blocking the transmembrane transport of polyamines into cancer cells and this has allowed us to determine if polyamine transport is an important component of cancer cell proliferation [23, 24]. This conjugate has been shown to be cytotoxic in cancer cells at relatively low concentrations. When this conjugate was tested in two MDR-positive human cancer cell lines, it was noted that the MDR variants were two- to fourfold more resistant to the cytotoxic effects compared to their parental cell lines [24]. Poly-SPM was also more potent in the MDR cells than in normal pulmonary artery smooth muscle cells [23]. These observations have led us to evaluate the effects of this novel Poly-SPM compound on MDR function in human cancer cells.

Materials and methods

Reagents

The sources of unlabeled drugs are as follows: doxorubicin (adriamycin), Adria Laboratories, Columbus, Ohio; vinblastine (velban), Eli Lilly, Indianapolis, Ind.; etoposide (vepesid) and taxol (paclitaxel), Bristol-Myers Squibb, Princeton, N.J.; cisplatin (platinol), Bristol Myers, Wallington, Conn.; PSC-833 (gift from Sandoz Pharmaceutical); and verapamil (isoptin), Knoll Pharmaceuticals, Whippany, N.J.. The polymeric polyamine-glutaraldehyde conjugate of spermine (Poly-SPM) was prepared in our laboratory and has been described elsewhere [23]. Briefly, the hydrochloride salt of the appropriate polyamine (0.1–1.0 mM) was dissolved in 1.0 ml 0.2 M phosphate-buffered saline (PBS) at pH 7.0 and the solution added to 1.0 ml of 0.2 M PBS containing glutaraldehyde (3% solution) at a pH of 7.0. The mixture was incubated at 37°C for 1 h, after which $NaBH_4$ (10 mg) was added to reduce the imine functions and the reaction mix was incubated at ambient temperature for 30 min. The reaction mixture was then dialyzed against 0.02 M PBS and this dialysate was lyophilized to afford the corresponding reduced polyamine-glutaraldehyde conjugate. Each batch of the conjugate produced was used within 24 h of synthesis and the structural characteristics were determined by both nuclear magnetic resonance and mass spectral analysis [23]. The MTT [3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyltetrazolium bromide] salt and all other chemicals, unless otherwise specified, were obtained from Sigma Chemical, St. Louis, Mo. All reagents were reconstituted in Dulbecco's PBS (Gibco, Grand Island, N.Y.) with final dilutions made in McCoy's 5A medium (GIBCO) supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), insulin (5 μg/ml), and 10% newborn calf serum (Gibco).

Cultured cell lines

The human MDR cell lines used in these experiments were the human uterine sarcoma cell line MES-SA and its doxorubicin selected MDR variant (Dx5), which is 50-fold resistant to doxorubicin and 30- to 60-fold cross-resistant to vinblastine, etoposide, and taxol when compared to the parental cell line MES-SA [27, 28]. These cell lines were grown in tissue culture flasks (Corning Glass Works, Corning, N.Y.) with McCoy's 5A medium supplemented with 2 mM glutamine, 5 mg/ml of insulin, 10% newborn calf serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin (Gibco). All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and subcultured every 5–7 days [25, 28].

Cytotoxicity assay

Cells grown in 75-cm^2 tissue flasks were harvested with 0.06 M EDTA, and cell number and viability were determined by a hemocytometer and Trypan Blue dye exclusion. Cells were seeded

in 96-well plates (Falcon, Becton Dickinson, Lincoln Park, N.J.) at 8000 cells per well and allowed to grow for 24 h at 37°C. Cells were then treated with either Poly-SPM (5.0–250 μM), doxorubicin, vinblastine, etoposide, and taxol alone (3.0 nM–3.0 μM), or with a combination of the SPM conjugate at a fixed concentration (25–50 μM) and the chemotherapeutic agents at concentrations described above. When the conjugate was used in combination with the chemotherapeutic agents, the control wells represented the conjugate alone, to eliminate any toxic effects the conjugate alone may have. The concentration of Poly-SPM resulting in 50% growth inhibition in the Dx5 cells is 68.0 ± 7.6 μM at 72 h. PSC-833 (0.5 μM) and verapamil (6.0 μM) were positive controls for MDR modulation. All cytotoxicity assays were initially done with and without 2.0 mM aminoguanidine, which had no effect on cytotoxicity by itself. This was added in order to assess whether amine oxidase products were involved, since aminoguanidine blocks the formation of these products. Cells were incubated for 72 h with these various drug combinations. Medium was then removed from each well, and 100 μl of fresh medium and 10 μl of MTT dye (5 mg/ml in PBS stock solution) were added to each well. Cells were incubated with MTT for 6 h, after which blue formazan crystals were dissolved in 0.1 N HCl in 2-propanol (100 μl per well). Within 30 min of dissolving the crystals, each plate was examined on a Dynatech MR580 Microelisa reader with a test wavelength of 570 nm and a reference wavelength of 634 nm [26, 27]. Each drug concentration was assayed in quadruplicate. The IC_{50} (the drug concentration resulting in 50% inhibition of MTT dye formation, compared to untreated or conjugate alone controls) was determined directly from semilogarithmic dose-response curves.

Accumulation studies

Accumulation studies were carried out in both the MES-SA and Dx5 cells grown in Falcon 35 × 10 mm sterile tissue culture plates (Becton Dickinson, Lincoln Park, N.J.). To each plate, 1×10^6 cells were added and allowed to attach overnight. The cells were then treated with the radioactive agent in the supplemented medium and placed in the incubator for 5–60 min at 37°C. Cells were treated with 4.0 μM [^{3}H]-vinblastine or 10.0 μM [^{3}H]-taxol in the presence and absence of unlabeled Poly-SPM (100 μM). Accumulation studies were done in the presence and absence of 2.0 mM aminoguanidine, which had no effect on these assays. At the indicated times, the plates were removed from the incubator, washed twice with 1.0 ml of ice-cold PBS, and then lysed with 1.0 ml of 4% SDS in deionized water. The lysed cells were then transferred to scintillation vials and 10 ml of Ecolite scintillation cocktail (ICN Biomedicals, Irvine Calif.) was added to each vial. Radioactivity was estimated on a Tri Carb C scintillation counter (Packard, Downer's Grove, Ill.). All the analyses for accumulation were done based on milligrams of cellular protein using the bicinchoninic acid method [25, 29].

Statistical analyses

Data are presented as the mean ± standard deviation. Differences between mean values were assessed using a one-way analysis of variance combined with Neuman-Kuels test, or using a non-parametric Mann-Whitney U test. The *a priori* level of significance was $P = 0.05$.

Results

The MDR modulation experiments were carried out using the MES-SA and Dx5 cells in the presence and absence of Poly-SPM. In these experiments the Dx5 cells were 40- to 50-fold resistant to doxorubicin, vinblastine, and etoposide, and 70-fold resistant to taxol, when compared to the parental cell line MES-SA. As a positive control for MDR modulation, PSC-833 (2.5 μM) lowered the IC_{50} of doxorubicin from 820 ± 30 μM to 18.0 ± 2.2 nM in the Dx5 cells, providing nearly complete reversal of resistance. The IC_{50} of the MES-SA cells was 15.0 ± 4.0 nM and no effect was seen with PSC-833. Poly-SPM at 50.0 μM was also able to reverse resistance to each of the above chemotherapeutic agents associated with MDR. A modulation ratio in the Dx5 cells (the IC_{50} of the chemotherapeutic agent alone versus the IC_{50} of the chemotherapeutic agent with Poly-SPM) was used to compare the effectiveness of Poly-SPM in reversing resistance to each of the chemotherapeutic agents. For doxorubicin (modulation ratio of 42) and vinblastine (modulation ratio of 36), Poly-SPM was able to almost completely reverse resistance in the MDR-positive cells, Dx5. Partial reversal of resistance was seen with etoposide (modulation ratio 29) and taxol (modulation ratio 25). These data are summarized in Table 1 and Fig. 1. With the addition of aminoguanidine (2.0 mM) to the taxol cytotoxicity assay, the modulation ratio was still 25 and aminoguanidine had no effect on the pattern of modulation (Table 2). The aminoguanidine results confirm that amine oxidase products were not involved in this effect. Poly-SPM did not sensitize the parental cell line MES-SA to these chemotherapeutic agents. The effect of Poly-SPM (50 μM) on cisplatin cytotoxicity (a non-MDR substrate) was minimal and resistance was not seen between the cells. The effect of Poly-SPM on reversal of resistance in the Dx5 cells was concentration-dependent between 25 and 50 μM. For doxorubicin, the modulation ratio decreased from 42 with 50 μM Poly-SPM to 17 with 25 μM; for vinblastine, 36 with 50 μM Poly-SPM to 6.1 with 25 μM; for etoposide, 29 with 50 μM Poly-SPM to 19 with 25 μM; and for taxol, 25 with 50 μM Poly-SPM to 5 with 25 μM. The data are summarized in Tables 1 and 2.

To further characterize the modulation of resistance exhibited by Poly-SPM in the MDR cells, Dx5, cellular accumulation studies were carried out with radioactive MDR substrates in the presence and absence of Poly-SPM. We have previously shown that maximum cellular accumulation of MDR substrates occurs within 30 min and all comparisons were therefore made at that time [25]. Poly-SPM (100 μM) was able to completely reverse the accumulation defect of [^{3}H]- vinblastine in Dx5 cells while having no effect on accumulation in MES-SA cells. This concentration of poly-SPM was used since maximal effects were seen at this concentration and, for a short 30-min incubation, this concentration was non-toxic to the cells. At 30 min, Poly-SPM increased [^{3}H]-vinblastine accumulation from 285 ± 24 pmol/mg protein to 790 ± 28 pmol/mg

Table 1 Modulation of resistance to multidrug resistant chemotherapeutic agents with a polymeric conjugate of spermine (*Poly-SPM*) (50 and 25 μM) in MES-SA and Dx5 cells. (*ND* not done)

Cell line	IC_{50} (nM) (control)	IC_{50} (nM) (50 μM Poly-SPM)	IC_{50} (nM) (25 μM Poly-SPM)	Modulation ratio[a]
Doxorubicin				
MES-SA	15.0 ± 4.0[b]	12.3 ± 4.2	16.3 ± 4.2	
Dx5	820 ± 30.0	19.5 ± 2.0[c]	48.0 ± 7.6	42
Vinblastine				
MES-SA	1.8 ± 0.4	1.5 ± 0.3	1.6 ± 0.2	
Dx5	92.0 ± 11.0	2.5 ± 0.2[c]	15.0 ± 3.5	37
Etoposide				
MES-SA	175.0 ± 15.0	153.0 ± 25.0	170.0 ± 30.0	
Dx5	7500.0 ± 450.0	251.0 ± 10.4[c]	381.0 ± 30.0	29
Taxol				
MES-SA	48.0 ± 4.0	40.0 ± 2.0	37.0 ± 6.0	
Dx5	3500.0 ± 325.0	140.0 ± 22.0[d]	620.0 ± 280.0	25
Cisplatin				
MES-SA	12.2 ± 2.0	9.8 ± 1.1	ND	–
Dx5	16.0 ± 5.1	14.4 ± 4.0	ND	

[a] Ratio of the IC_{50} (concentration which inhibits 50% growth at 72 h) of the chemotherapeutic agent alone versus the IC_{50} in the presence of 50 μM of the Poly-SPM conjugate in the Dx5 cells
[b] Mean ± SD of six determinations
[c] $P < 0.05$ for Dx5 control versus Dx5 with 50 μM Poly-SPM
[d] $P < 0.01$ for Dx5 control versus Dx5 with 50 μM Poly-SPM

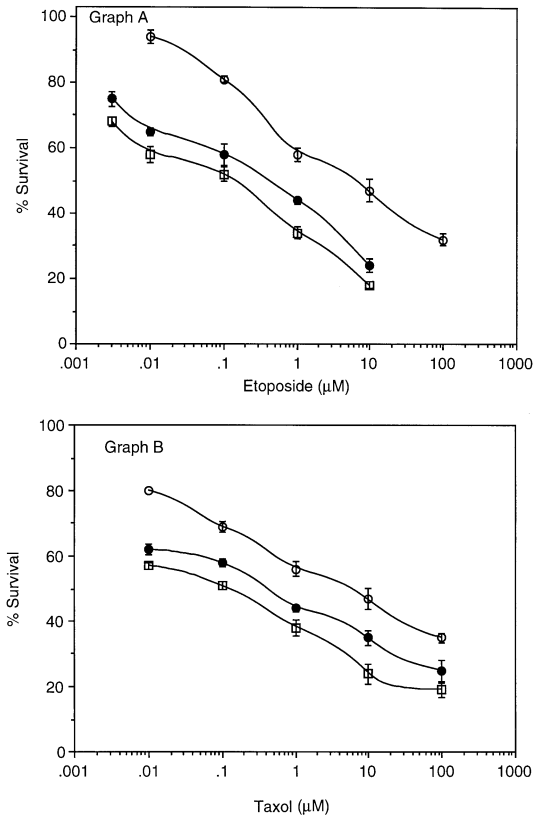

Fig. 1 Cytotoxicity assay (72 h) of etoposide (**A**) or taxol (**B**) in the presence and absence of a polymeric conjugate of spermine (Poly-SPM). (○) Dx5 control; (●) Dx5 + 25.0 μM Poly-SPM; (□) Dx5 + 50.0 μM Poly-SPM

protein in the Dx5 cells ($P < 0.05$, see Fig. 2A). Poly-SPM (100 μM) was also able to completely reverse the accumulation defect of [^{3}H]-etoposide when compared to the parental cell line MES-SA, as shown in Fig. 2B. Taxol experiments were carried out at 30 min in the presence and absence of both Poly-SPM (100 μM) and aminoguanidine (2.0 mM). The latter had no effect on the cellular accumulation of taxol or the effects of Poly-SPM on taxol accumulation. Poly-SPM (100 μM) was able to completely reverse the 4.4-fold accumulation defect seen in the MDR-positive cells, Dx5 (Fig. 3).

Cellular accumulation studies were also carried out with radiolabelled MDR substrates in the presence and absence of the ornithine decarboxylase inhibitor, DFMO, which has been shown to increase the activity of the polyamine transporter as a result of decreased polyamine synthesis. With [^{3}H]-vinblastine, cellular accumulation in the MDR cells was 290 ± 18 pmol/mg cellular protein in the controls and 198 ± 11 in the Dx5 cells pretreated for 24 h with 5.0 mM DFMO (control versus DFMO, $P < 0.05$). This same trend was observed with [^{3}H]-taxol. Following 24-h pretreatment with DFMO at a concentration of 2.5 mM, decreased cellular accumulation of taxol from 421 ± 31 pmol/mg cellular protein in the controls to 370 ± 27 pmol/mg cellular protein in the DFMO-treated Dx5 cells (control *versus* DFMO, $P < 0.05$) was observed. With 24-h pretreatment at a higher concentration of DFMO (5.0 mM), the cellular accumulation dropped to 217 ± 29 pmol/mg cellular protein (control versus

Table 2 Effect of 2.0 mM aminoguanidine (AG) on modulation of taxol resistance with SPM conjugate (50 μM).

Cell line	Control (nM)	Control + AG (nM)[a]	Poly-SPM (nM)	Poly-SPM + AG (nM)
MES-SA	48.0 ± 4.0	40.0 ± 2.0[b]	39.5 ± 3.5	37.0 ± 6.0
Dx5	3500.0 ± 285.0	3300.0 ± 325.0	140.0 ± 22.0	150.0 ± 12[c]

[a] Mean IC_{50} values ± SD, $n = 4$ separate determinations (taxol cytotoxicity assay carried out for 72 h)
[b] MES-SA taxol control with AG versus Dx5 taxol control with AG, $P < 0.05$
[c] Dx5 taxol with AG control versus Dx5 taxol with AG and SPM, $P < 0.05$

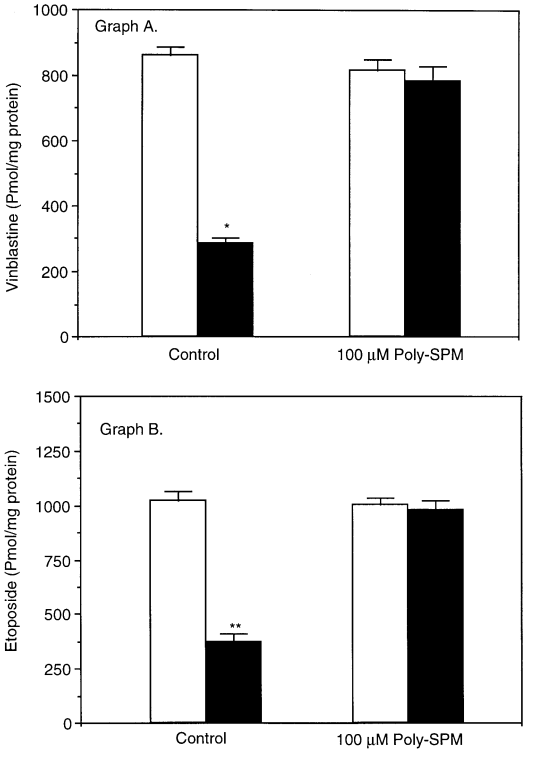

Fig. 2 Cellular accumulation at 30 min of 4.0 μM [^{3}H]-vinblastine (**A**) and 10.0 μM [^{3}H]-etoposide (**B**) in (□) MES-SA and (■) Dx5 cells with and without 100 μM Poly-SPM. (Mean ± SD, $n = 8$ for each graph) *, $P < 0.05$, MES-SA control versus Dx5 control; **, $P < 0.05$, MES-SA control versus Dx5 control

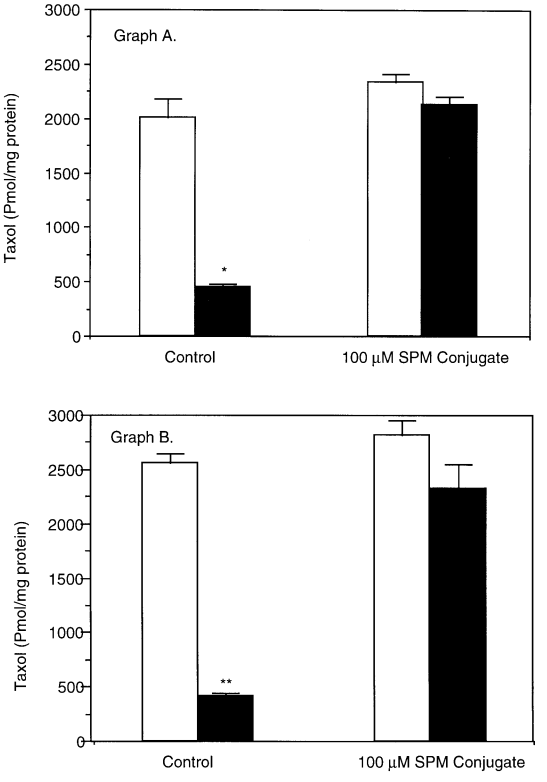

Fig. 3 Cellular accumulation at 30 min of (10.0 μM) [^{3}H]-taxol in the absence (**A**) and presence (**B**) of 2.0 mM aminoguanidine in (□) MES-SA and (■) Dx5 cells with and without 100 μM Poly-SPM. (Mean ± SD, $n = 8$ for each graph) *, $P < 0.05$, MES-SA control versus Dx5 control; **, $P < 0.01$, MES-SA control versus Dx5 control

DFMO, $P < 0.01$). There was no effect on cellular accumulation of [^{3}H]-vinblastine or [^{3}H]-taxol in the parental cell line following pretreatment with DFMO (Fig. 4).

Discussion

We have previously described a novel polymer of spermine which inhibits the inward transport of polyamines in pulmonary artery smooth muscle cells as well as in two cancer cell lines [23, 24, 29, 30]. This Poly-SPM has been shown to be particularly toxic to cancer cell lines and its effect is reversed in the presence of excess polyamines. These results are not surprising, in that neoplastic cells exhibit higher polyamine contents than normal cells and this is associated with the higher proliferation rates observed in cancer cells [31]. Most of the previous studies have focused on structural modification of naturally occurring polyamines with the aim of altering polyamine metabolism or function once inside the cancer cell. Promising compounds currently in various phases of clinical development include the polyamine analog N^1, N^{11}-diethylnorspermine (DENSPM) and a SAMDC inhibitor, CGP-48664. DENSPM has been shown to rapidly deplete intracellular polyamine pools by down-regulating ODC and

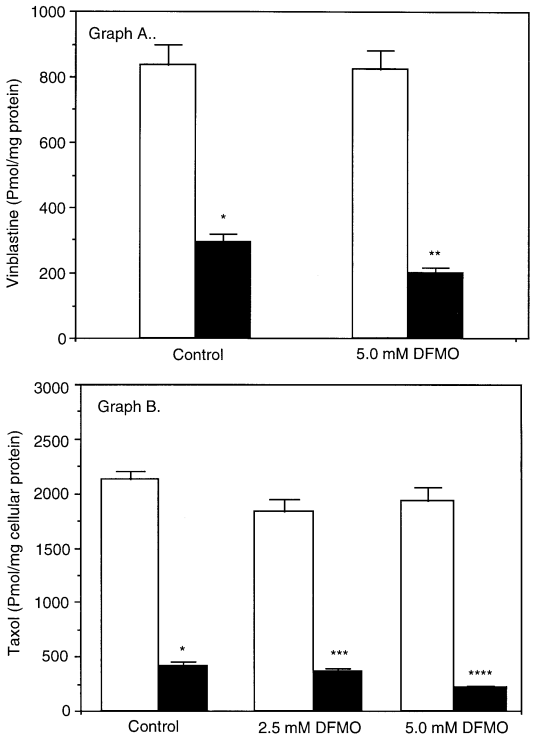

Fig. 4 Cellular accumulation at 30 min of 4.0 μM [^{3}H]-vinblastine (**A**) and 10.0 μM [^{3}H]-taxol (**B**) in (□) MES-SA and (■) Dx5 cells with and without α-difluoromethy-ornithine (DFMO) at 2.5 or 5.0 mM. (Mean ± SD, $n = 6$ for each graph) *, $P < 0.05$, MES-SA control versus Dx5 control; **, $P < 0.05$, Dx5 control versus Dx5 + 5.0 mM DFMO; ***, $P < 0.05$, Dx5 control versus Dx5 + 2.5 mM DFMO; ****, $P < 0.01$, Dx5 control versus Dx5 + 5.0 mM DFMO

SAMDC activity as well as up-regulating N^1-acetyltransferases, which catabolize polyamines [32]. CGP-48664 is currently in phase I clinical studies, and is an inhibitor of SAMDC [33]. Other researchers have synthesized polyamine analogs which are transported into cancer cells and then act as DNA alkylating agents. These compounds utilize the polyamine transporter to enter the cancer cells, targeting therapy more specifically to neoplastic tissues. Two of these analogs, the N^1 and N^8-aziridinyl spermines show significant preferential toxicity in cancer cells and offer another approach to cancer chemotherapy [34]. We have synthesized polymers of polyamines which inhibit transport of polyamines into cancer cells and also show significant cytotoxic effects in two cancer cell lines [23]. These compounds are unique in that MDR-positive cancer cells appear to be more resistant to these polymers than their parental cell lines. Preliminary data have also shown that the cytotoxic effect of these compounds can be blocked with excess free polyamine, providing evidence that polyamine transport may be involved [23, 24]. Further work in our laboratory is ongoing to evaluate this interaction further.

We have shown that in an MDR-positive human uterine sarcoma cell line Dx5, Poly-SPM can function as an MDR modulator, reversing resistance to doxorubicin, vinblastine, etoposide, and taxol. In the parental cell line, MES-SA, Poly-SPM had no effect on the cytotoxicity of these chemotherapeutic agents. Since the primary difference between the Dx5 and MES-SA cell lines is the expression of P-glycoprotein, cellular accumulation experiments in the presence and absence of Poly-SPM were also performed. Poly-SPM was able to reverse the cellular accumulation defect of vinblastine and taxol in the Dx5 cells, increasing the cellular levels of these compounds to those observed in the parental cell line, MES-SA. This result supports the concept that Poly-SPM is interacting in some way to inhibit the function of P-glycoprotein. When the Dx5 cells were pretreated with the ODC inhibitor, DFMO, the opposite effect was observed; DFMO increased the function of the polyamine transporter but also caused an even further decrease in the cellular contents of vinblastine and taxol. This effect was again preferentially seen in the MDR-positive Dx5 cells, with no effect observed in the parental cell line. These studies stongly suggest a potential link between polyamine inward transport and the efflux function of P-glycoprotein, wherein inhibition of the polyamine inward transporter(s) results in decreased P-glycoprotein drug efflux function.

Other polyamine inhibitors which have been studied in MDR cancer cell lines have exhibited no resistance and in, some cases, collateral sensitivity to these compounds. Both the DENSPM and CGP-48664 compounds have been shown to have high activity in an MDR melanoma cell line [32]. Other investigators have analyzed an N^1,N^{12}-bis(ethyl)spermine compound in an MDR breast cancer cell line (MCF-7) and demonstrated equal sensitivity among drug resistant and parental cell lines. These compounds exert their primary cytotoxic effect on polyamine synthesis. MDR cells have exhibited ODC:SAMDC ratios of from 7:1 to 9:1 versus parental cell lines ratios of 2:1 [35]. This is most likely due to increased ODC activity observed in MDR versus parental cells, and is one of the reasons why the MDR cells are sensitive to DENSPM. Poly-SPM specifically inhibits polyamine uptake and the MDR cells, Dx5, were resistant to its toxic effects when compared to the parental MES-SA cells [24].

Preliminary data have shown that the uptake kinetics of polyamines in the Dx5 cells exhibit a V_{max} for putrescine and spermine which is two to three-fold lower than that seen in the parental MES-SA cell line. In contrast, the K_m is 1.5- to 4-fold higher in the Dx5 cells compared to the MES-SA cell line [24]. Further studies are ongoing to verify these potential differences. Another study analyzing the human leukemia cell line, K562, and its MDR variant, K562MDR, revealed that the V_{max} was lower in the MDR cells but no change in the K_m was observed between the cell lines [31]. This

difference can be explained by the fact that the MDR cells had a slower doubling time than the parental cell line. The results from this study showed that the MDR cells had lower levels of all polyamines than the parental cell line, indicating that the cells were defective in both polyamine uptake and biosynthesis. In our study, the doubling time of the MES-SA and Dx5 cells is similar, therefore, the observed differences between the parental and MDR-positive cell lines cannot be explained in this way. One difference between our study and the study mentioned above may be that the Dx5 cell line we used has a lower overall level of resistance (40- to 50-fold resistance to doxorubicin) compared to the K562MDR cells, which are 75- to 100-fold resistant to doxorubicin.

The MDR modulation data and the polyamine uptake data suggest a potential functional linkage between the MDR transporter and the polyamine transporter. It has been shown that the polyamine transporter does exhibit biphasic responses in cancer cell lines, although the relationship between this observation and the MDR phenotype is unknown [36]. Our data suggest that Poly-SPM can function as a potent modulator of MDR and that Poly-SPM is acting at an extracellular site [23]. Most of the currently known MDR modulators are thought to block the P-gp transporter in a competitive manner and are either transported themselves by P-gp or simply bind to the membrane transporter. Structure-activity relationships (SARs) for MDR modulators have been studied for several classes of drugs, and some general principles governing the binding and export of compounds by P-gp have emerged [21]. For most modulators of MDR, hydrophobic ring moieties and tertiary amino groups are important characteristics [37]. Several MDR modulators, including the cyclosporines, do not fit these simple SARs. Poly-SPM also does not fit into any of the known SARs for MDR substrates or modulators. Several observations could explain our current findings. First, the interaction of Poly-SPM in the MDR cells could be simple steric hindrance of P-gp, with Poly-SPM binding to the polyamine transporter. This would explain the MDR modulation data with Poly-SPM, but does not account for the increased activity of P-gp following DFMO pretreatment, nor the differences observed in polyamine uptake kinetics in the MDR *versus* parental cell lines. An alternative, and perhaps more appealing hypothesis, would be that there is a functional link between these two similar ATP-dependent transporters. This phenomenon has been observed with respect to P-gp and the cystic fibrosis transmembrane conductance regulator protein (CFTR). It has been shown that in normal colonic epithelial cells, induction of the MDR transporter (P-gp) can cause a down-regulation of CFTR [38]. Since many of these ATP-dependent transporters on cell membranes are members of the ATP-binding cassette superfamily of active transporters, more functional interactions may exist among these transporters [39].

The clinical use of MDR modulators in cancer patients has primarily focused on giving very high doses of the modulator to the patient. When these modulators are combined with the chemotherapeutic agents, significant toxicities have been noted. The MDR modulators not only block the function of P-gp in the cancer cells, but may also block the activity seen in normal tissues including the liver, kidney, hematopoietic cells, and the blood-brain barrier. This has resulted in enhanced as well as unexpected toxicities in several clinical trials [40]. Alterations in the pharmacokinetic and toxicity profiles of these chemotherapeutic agents may also be the result of having to achieve very high levels of the MDR modulators in patients in order to reverse drug resistance. Interactions between the modulators and the chemotherapeutic agents may, in part, be unrelated to MDR expression in normal tissue. A new approach to inhibiting the MDR transporter may help not only in blocking the activity of P-gp in human tumors, but in limiting toxicities seen with high doses of these classic MDR modulators (i.e., verapamil and cyclosporine). The utilization of an inhibitor of MDR which functions to inhibit the P-glycoprotein transporter by a unique mechanism, may allow for more effective and less toxic therapies. Detailed toxicology studies with Poly-SPM in the presence and absence of chemotherapeutic agents (eg., taxol, doxorubicin, etoposide, and vinblastine) are ongoing.

We have described a novel inhibitor of P-gp, although the interaction is currently unknown. Our data suggest that the inhibition of P-gp is through an interaction with the polyamine transporter; studies are ongoing to further prove this point. This Poly-SPM inhibits polyamine transport in cancer cells and causes significant cytotoxicity [23]. Poly-SPM has also been shown to preferentially sensitize an MDR-postive human sarcoma cell line to antineoplastic agents, which are substrates for P-glycoprotein. These initial data suggest a potential functional link between these two transporters, which may help further our understanding of the regulatory elements of each transporter as well as provide a novel way to inhibit the MDR transporter.

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