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Original Paper

Modulation of *cis*-Diamminedichloroplatinum (II) Accumulation and Cytotoxicity by Spermine in Sensitive and Resistant Human Ovarian Carcinoma Cells

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The effect of spermine (Sp), a natural polycationic amine, on cisplatin (CDDP) sensitivity and accumulation of a human ovarian CDDP-sensitive cell line (2008) and its resistant variant (C13*) was investigated. Survival was also studied. The C13* cells were approximately 20-fold resistant to CDDP, yet were found to be just as sensitive to Sp as 2008 cells. When Sp was concurrently added with CDDP to the colony-forming assay, the IC_{50} dose was approximately 3-fold lower than that of CDDP alone. This decrease was the result of a synergistic interaction, as assessed by median effect analysis. The incubation of cells with the approximate IC_{50} dose of Sp for 1–8 h indicated that this synergism could be due to stimulation of CDDP accumulation, showing maximal uptake after 4 h of Sp exposure. This stimulation may be the result of a modulation of cellular membrane permeability by Sp, as assessed by the accumulation of [^{3}H]mannitol. Exposure to Sp concentrations active on CDDP uptake also significantly increased [^{3}H]mannitol accumulation in both cell lines. The triamine spermidine (Spd) did not significantly affect either the sensitivity of the two cell lines or CDDP and [^{3}H]mannitol accumulation. These results suggest that Sp is a positive modulator of CDDP uptake, and thus of its cytotoxicity, even in resistant cells, where the phenotype is partly due to a CDDP accumulation defect.

Key words: cisplatin, drug resistance, drug transport, cell membrane permeability, polyamines

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INTRODUCTION

CISPLATIN (CDDP) is one of the most widely used and potent drugs for the treatment of solid tumours, such as human ovarian and testicular carcinomas. However, resistance to this drug is often encountered *in vitro* and *in vivo*. Postulated mechanisms of CDDP resistance include decreased drug accumulation, increased levels of the intracellular thiols and increased DNA repair [1]. The decrease in CDDP accumulation associated with resistance is usually modest compared with the level of resistance, but it has been shown to appear early in the development of resistance *in vivo* [2]. Many studies have been undertaken to assess how CDDP enters cells and how this entry could be modulated in order to overcome this resistance phenotype. Along with studies supporting a passive diffusion role in CDDP accumulation [3–5], some researchers have shown that CDDP uptake can be modulated by different physiological and pharmacological conditions [6]. A working model for CDDP accumulation has therefore been suggested in which it is postulated that CDDP enters the cell not only by passive diffusion but also by transport through a gated ion channel [6]. All studies in this area attempt to clarify this mechanism and to identify structural or functional alterations responsible for the accumulation defect.

Spermine (Sp) and its precursors spermidine (Spd) and putrescine are naturally-occurring polyamines essential for normal cell growth and differentiation [7]. However, not only their depletion but also their addition at high concentrations to culture medium inhibits the growth of normal

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and tumour cell lines [8, 9], even if their degradation into cytotoxic aldehydes is prevented by the addition of aminoguanidine (AG), an inhibitor of copper-dependent amine oxidases contained in bovine serum [10]. The cytoxicity of polyamines, and of Sp in particular, appears to be a result of many effects [11, 12], but some intracellular actions of polyamines have not been clearly defined. Polyamines are also thought to exert cell-membrane effects by interacting with membrane components such as phospholipids (PL) and proteins. These interactions could regulate the activity of membrane-bound enzymes [13] and affect the transport of ions, metabolites and other molecules.

In the light of these findings, and in view of the fact that the cellular pharmacology of CDDP can be affected at different levels, we have investigated the effect of exogenous Sp on CDDP accumulation and sensitivity in a CDDP-sensitive human ovarian carcinoma cell line, 2008 cells and in its resistant variant, C13* cells.

MATERIALS AND METHODS

Drugs and chemicals

CDDP was a generous gift of Andrulis Pharmaceuticals Co. (Beltsville, Maryland, U.S.A.). Sp, other polyamines and AG were purchased from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.).

Cell lines

The 2008 cell line, established from a patient with serous cystadenocarcinoma of the ovary and the CDDP-resistant C13* subline, generated as previously described [14], were grown as monolayers in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, Maryland, U.S.A.) containing 10% heat-inactivated fetal bovine serum (Gibco/BRL, Gaithersburg, Maryland, U.S.A.) and 50 μg/ml gentamicin sulphate (Sigma). Cultures were equilibrated with humidified 5% CO_2 in air at 37°C. All studies were performed with *Mycoplasma* negative cells as determined with the *Mycoplasma* T.C. detection kit (Gen-Probe, San Diego, California, U.S.A.). Clonogenic assays on plastic dishes were conducted as previously described [15]. Briefly, the cells were seeded at a cell density of 250 cells/dish in 60 mm tissue culture dishes and allowed to attach overnight; they were then exposed to CDDP or Sp, or to combinations of the two drugs, at the constant ratio of their IC_{50} values. When Sp was present in the medium, 1 mM AG was added to prevent Sp metabolism into cytotoxic aldehydes by bovine serum amine oxidases. AG did not show any appreciable effect either on CDDP cytotoxicity or as a single agent. To assess whether the cytotoxic effects of CDDP and Sp were antagonistic, additive or synergistic, median effect analysis was conducted using a computer program (Elsevier-Biosoft, U.K.), as described by Chou and Talalay [16].

CDDP accumulation

One-hour CDDP accumulation experiments were performed with subconfluent monolayers as previously described [17]. Experiments were performed in the cell culture medium without serum or antibiotics. In order to determine the pre-incubation-time effect on CDDP uptake, the plates were incubated with the approximate IC_{50} concentration of Sp in a colony-forming assay over different periods, 100 μM CDDP being added for the final hour. In polyamine concentration-dependent experiments, the cells were exposed to increasing concentrations of polycations for the time of maximal CDDP uptake, as measured in timing experiments. Incubations were terminated by washing four times with ice-cold phosphate-buffered saline (PBS) and cells were scraped into 0.1% Triton-X-100 in 0.1 N HCl and frozen until use. After thawing and sonication, cell-associated platinum was measured with a Perkin-Elmer Model 5000 (Perkin Elmer, Norwalk, Connecticut, U.S.A.) atomic absorption spectrophotometer equipped with an HGA 400 graphite furnace accessory. Each sample was run in duplicate.

[^{3}H]*Mannitol accumulation*

Membrane passive permeability studies were carried out on subconfluent cells seeded into 6-well plates and treated, after medium aspiration, with 1 ml RPMI 1640 medium containing 1 mM [^{3}H]mannitol (1 μCi/ml) (Sigma) [18, 19] with or without Sp in the same conditions as described for CDDP accumulation, the permeability marker being added for the final hour of incubation. At appropriate intervals, the medium was aspirated and the wells rapidly washed four times with ice-cold PBS. The cells were digested overnight with 1 ml of 1 N NaOH. A 0.7 ml aliquot was mixed with 7 ml of acidified liquid scintillation cocktail and radioactivity measured on a Tri-Carb 460 C liquid scintillation counter (Camberra–Packard).

Measurement of polyamines

The cells were allowed to attach overnight and grown in the presence of the approximate IC_{50} dose of Sp for a period of 96 h. At different times within this period, cells were harvested, pelleted, washed with PBS and extracted with 0.25 M $HClO_4$ for determination of intracellular polyamine content, essentially according to Seiler and Knodgen [20], after dansylation, as previously described [21]. The acid-insoluble cell pellet was resuspended in 0.3 M NaOH, and aliquots were used for the determination of protein.

Protein estimation

Proteins were estimated by the method of Lowry and associates [22].

RESULTS

The sensitivity of 2008 and C13* cells, respectively, to CDDP and Sp alone is shown in Figure 1. The IC_{50} dose for CDDP was 0.040 ± 0.003 μM in 2008 cells and 0.824 ± 0.049 μM in C13* cells, corresponding approximately to a 20-fold resistance, yet the two cell lines were found to be equally sensitive to Sp, to which IC_{50} dose was 83 ± 11 μM in 2008 cells and 75 ± 5 μM in C13* cells. When the combination of the two drugs was added to the assay, their concentration being increased in proportion to their IC_{50} values, cell survival decreased significantly with a new IC_{50} dose for CDDP of 0.014 μM in 2008 cells and 0.265 μM in C13* cells, representing approximately a 3-fold decrease ($P < 0.01$) in both cell lines (Figure 2). Median effect analysis of the interaction between these two drugs gave a synergistic response ($CI < 1$) for all levels of colony inhibition in 2008 cells and when colony formation was inhibited by more than 20% in C13* cells (Figure 3). This synergistic combination yielded a CI_{50} (combination index 50) of 0.61 ± 0.15 in 2008 cells and 0.7 ± 0.07 in C13* cells.

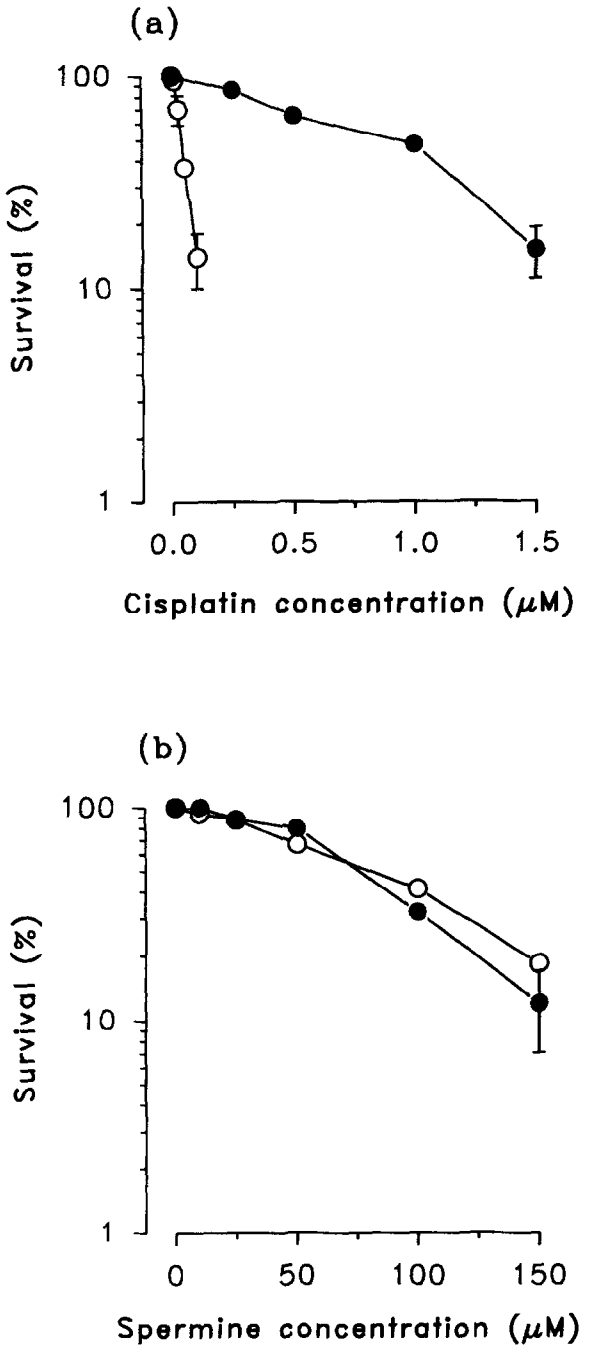

Figure 1. Cytotoxicity of CCDP alone (a) and of Sp alone (b). Colonies of 2008 cells (○) and C13* cells (●) were counted after 10 days of incubation. Each point represents mean values ± SE of three separate experiments each conducted with triplicate plates.

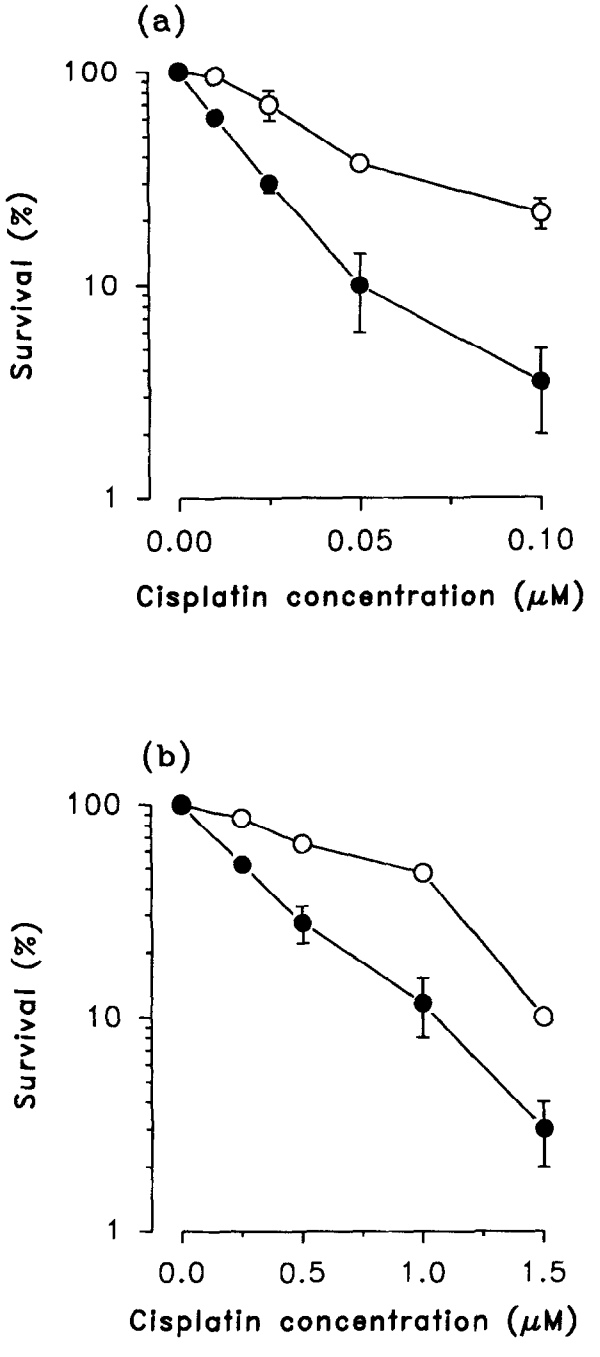

Figure 2. Dose-response curve for the combination of CDDP and Sp (●) plotted against increasing concentrations of CDDP alone (○) in the 2008 cell line (a) and in the C13* cell line (b). Each point represents mean values ± SE of three separate experiments each conducted with triplicate plates.

In order to explain the synergism observed between CDDP and Sp, we investigated whether Sp first might affect CDDP accumulation. As shown in Figure 4, exposure to the approximate IC_{50} dose of Sp over different periods produced the maximal effect on 1-h CDDP accumulation after 4 h of total Sp exposure in both cell lines and then returned to the control value after 6–8 h. The uptake increased up to 53 ± 8% in 2008 cells and 38 ± 5% in C13* cells; both increments were significantly higher ($P < 0.05$) than the respective controls, for which accumulation was 312 ± 16 and 171 ± 7 pmol/mg of protein. In accumulation-defective C13* cells, CDDP uptake remained significantly lower ($P < 0.05$) both at 2 h and at 4 h with respect to the 2008 parental cell line.

When CDDP uptake was assessed in the presence of increasing Sp concentrations for 4 h, being the time of maximum CDDP accumulation, increments became significant at concentrations around the IC_{50} dose, with a maximum at 100–150 μM and no further stimulation above this concentration (Figure 5).

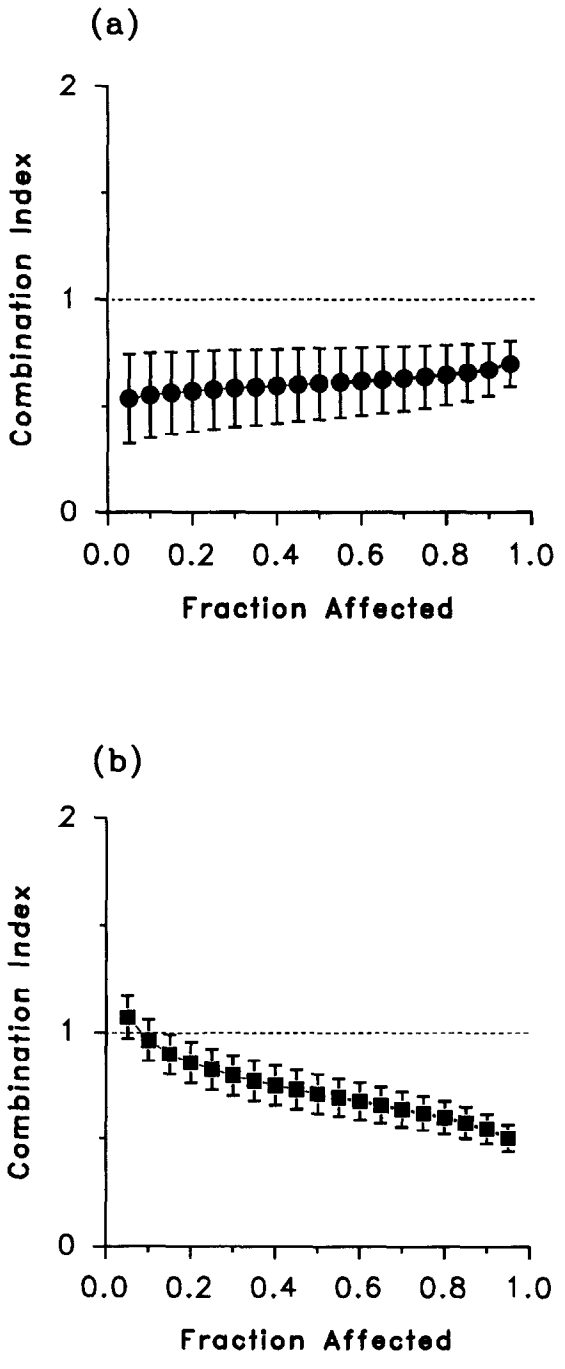

Figure 3. Plot of the CI as a function of cell kill for the interaction between CDDP and Sp for 2008 (a) and C13* (b) cell lines. Each data point represents the mean ± SE of a minimum of three experiments performed with triplicate cultures.

Of the Sp structural analogues tested, only 1,12-diaminododecane (1,12-DD), a diamine which has the same molecular length as Sp (approximately 20 Å) but has no internal amine groups, was able to produce a 30% increase in CDDP uptake in 2008 cells and 20% in C13* cells at 300 μM. This effect could probably explain the 3.2-fold and the 2-fold potentiation of CDDP cytotoxicity that 1,12-DD also provoked in 2008 and C13* cells, respectively (data not shown). Triethylenetetramine (TT), which has internal and external amine groups like Sp but is almost as long as

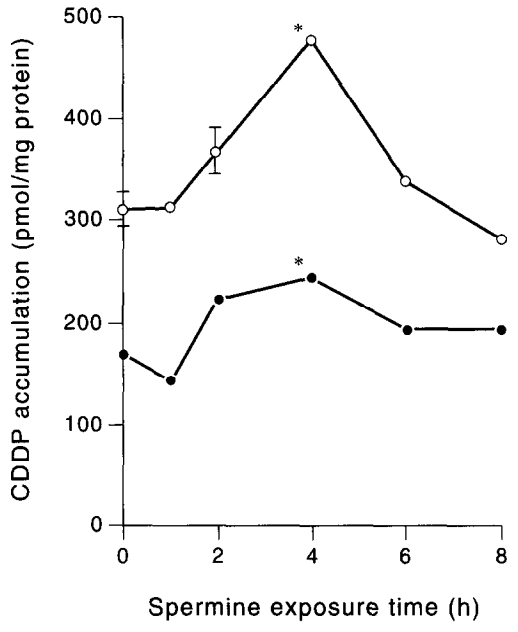

Figure 4. Effect of Sp pre-incubation time on 1-h CDDP accumulation in 2008 (○) and C13* cells (●). Each data point represents mean ± SE of a minimum of three experiments performed with duplicate cultures. *$P < 0.05$ by Student's t-test versus controls.

the triamine spermidine (Spd) (approximately 14 Å), a natural precursor of Sp, was as ineffective as Spd on both cell lines (Figure 6). Likewise, Spd was less cytotoxic on these cell lines since colony formation was reduced to only 70% of control values even in the presence of 2mM Spd (data not shown).

To find out whether the modulation of CDDP accumulation by Sp could be explained by an effect on the permeability of cellular plasma membrane, the cells were

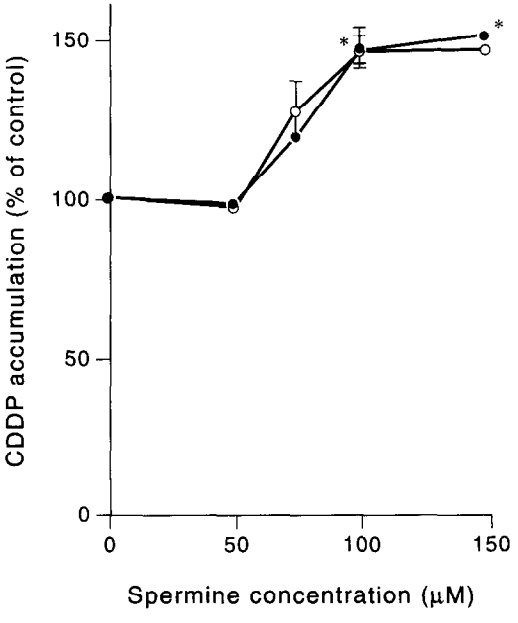

Figure 5. Effect of the 4-h exposure to increasing Sp concentrations on 1-h CDDP accumulation in 2008 (○) and C13* cells (●). Each data point represents mean ± SE of a minimum of two experiments performed with duplicate cultures. *$P < 0.05$ by Student's t-test versus controls.

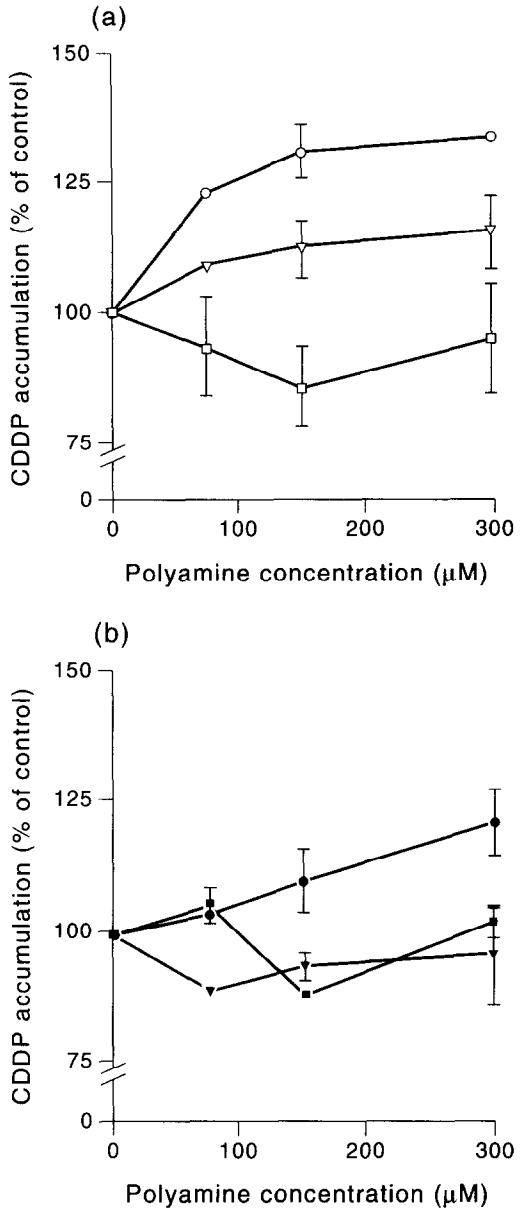

Figure 6. Effect of 4-h exposure to increasing concentrations of Sp structural analogues on 1-h CDDP accumulation. 2008 cells (a) and C13* cells (b) were treated with the indicated concentrations of 1,12-diaminododecane (○, ●), triethylenetetramine (▽, ▼) and spermidine (□, ■). Each data point represents mean ± SE of a minimum of two experiments performed with duplicate cultures.

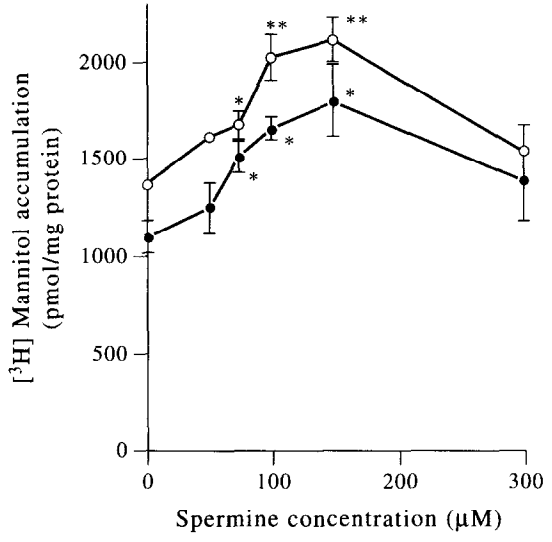

Figure 7. Effect of 4-h exposure to increasing Sp concentrations on 1-h [^{3}H]mannitol accumulation in 2008 (○) and in C13* cells (●). Each data point represents mean ± SE of a minimum of three experiments performed with duplicate cultures. *$P < 0.05$; **$P < 0.01$ by Student's t-test versus controls.

incubated with an increasing concentrations of Sp for 4 h and [^{3}H]mannitol, a compound usually used as a marker of this process, was added for the final hour in order to reproduce the same conditions of CDDP uptake. Mannitol is a hydrophilic molecule that is thought to penetrate biological membranes mainly by way of aqueous channel-like pores and not by way of lipoid regions [18, 19]. Figure 7 shows that the basal permeability of 2008 cells to [^{3}H]mannitol was significantly higher (t-test) than CDDP-resistant cells, as previously reported [23], with 1377 ± 34 versus 1102 ± 79 pmol/mg ($P < 0.05$), respectively. Treatment of 2008 cells with 150 μM Sp brought the maximal accumulation up to 2107 ± 109 pmol/mg ($P < 0.01$), an increase of $53 \pm 8\%$ over the control value, and up to 1797 ± 181 pmol/mg ($P < 0.05$) in C13* cells, which represents an enhancement of $63\% \pm 5\%$ over untreated C13* cells, with the difference between the two cell lines maintained. In both cell lines, [^{3}H]mannitol uptake was also significantly increased by 75 μM and 100 μM Sp for the same exposure time, indicating a correlation between CDDP uptake and permeability marker accumulation. Spd failed to modulate this process, either at micro- or millimolar concentrations. 1,12-DD and TT had approximately the same effect on mannitol accumulation as was observed for CDDP uptake (data not shown), suggesting that the particular molecular length and the distance between the external amine groups play a major role in this process.

Once inside the cells, exogenous Sp is able to perturb the intracellular polyamine content affecting their growth status, as widely reported [7]. Since these cell lines have shown the same sensitivity to Sp, we investigated whether CDDP-sensitive and -resistant cell lines had a similar polyamine content and how this pool was modified by exposure to the IC_{50} dose of Sp over different periods. Table 1 shows that, despite a lower level of polyamines in C13* cells at 8 h, the basal polyamine content of the two cell lines was similar at 24 h and beyond, and that, approaching confluence, their Spd content in particular decreased. It has been reported that Spd depletion can be explained as a result of its excretion into the culture medium as a response of the cells to the inhibition of growth rate due to many factors, including increased cell density and pharmacological agents [24, 25]. According to these findings, exogenous Sp, in the reported conditions, mainly affects the Spd content of both cell lines, increasing the reduction of this triamine to a level significantly lower than that of untreated controls at 24 h and for most of the time of the survival assay.

DISCUSSION

We have shown that the addition of Sp to the culture medium, in the conditions at which extracellular and intra-

Table 1. Effect of spermine on polyamine profile of 2008 and C13 cells*

Exposure time (h)		Intracellular polyamine content (nmol/mg protein) Putrescine	Spermidine	Spermine
2008 cells				
4	—	3.88 ± 1.23	6.65 ± 1.86	10.3 ± 1.71
	+Sp	3.92 ± 2	6.74 ± 1.1	15.45 ± 0.65
8	—	3.91 ± 1.7	7.35 ± 1.96	11.55 ± 1.55
	+Sp	2.74 ± 1	4.63 ± 1.47	12.15 ± 0.5
24	—	2.55 ± 1.7	5.6 ± 0.65	9.4 ± 0.9
	+Sp	2.76 ± 1.6	2.84 ± 0.76*	16 ± 1.45
48	—	2.1 ± 0.5	3.1 ± 0.38	9 ± 1.6
	+Sp	1.92 ± 0.42	1.18 ± 0.2*	12.9 ± 0.9
96	—	N.D.	1.7 ± 0.2	6.7 ± 1
	+Sp	N.D.	0.8 ± 0.1*	10 ± 2
C13* cells				
4	—	2.71 ± 1	7.98 ± 1.73	10.10 ± 0.3
	+Sp	2.21 ± 1.4	6.3 ± 0.9	13.45 ± 0.2
8	—	1.96 ± 1.2	6 ± 2.1	7.6 ± 1.9
	+Sp	1.33 ± 0.5	4.6 ± 1.4	10.8 ± 0.5
24	—	2.13 ± 1	5.42 ± 0.25	10.7 ± 0.2
	+Sp	2.2 ± 0.5	2.15 ± 0.85*	13.35 ± 0.4
48	—	2 ± 1	2.93 ± 0.13	8.55 ± 1.1
	+Sp	1.9 ± 0.8	0.93 ± 0.06**	12.4 ± 1.4
96	—	N.D.	2.1 ± 0.2	8.2 ± 1.30
	+Sp	N.D.	0.9 ± 0.1*	9 ± 1.5

Values are means ± SE of two to three experiments. *$P < 0.05$; **$P < 0.01$ by Student's *t*-test versus controls. N.D., not determined.

cellular copper-dependent polyamine oxidases are inactive, inhibits the colony formation of human ovarian carcinoma cell lines and that this inhibition is synergistic to that caused by CDDP. We have also shown that this effect of Sp can be ascribed to an increase in CDDP accumulation so that more drug is available for cell damage. The observed synergism is consistent with the finding of Oredsson and associates [26], who reported that 2-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase (ODC), decreased the cytotoxicity of CDDP against 9L rat brain tumour cells, but that this phenomenon was reversed by restoring the intracellular polyamine level with an exogenous supplement. In contrast, Allen and Natale [27] showed that DFMO and thus polyamine depletion increased the cytotoxicity of CDDP in P3J lymphoma cells. Again, Roizin-Towle [28] concluded that millimolar concentrations of putrescine or 15 μM Sp concurrently administered with CDDP reduced the cytotoxic action of CDDP on V79 hamster cells, which they showed to be less sensitive to Sp alone and more sensitive to Spd alone, unlike our cell lines; also, they used only one concentration in combination with CDDP for a short time, whereas we used increasing concentrations of Sp and CDDP at a constant ratio of their IC_{50} doses and for a longer exposure time. All these studies tend to show that the effect of CDDP in the presence of exogenous or endogenous polyamine is dependent on the cell line and on the type of protocol followed.

Furthermore, we have shown that Sp, but not Spd, is able to increase CDDP uptake in a way that seems to be molecular-structure specific and this stimulation, along with other possible intracellular effects, could account, at least in part, for the synergism in both cell lines.

We also show a good parallelism between CDDP accumulation and the positive modulation of plasma membrane permeability by Sp, thus indicating that, in this mechanism, passive diffusion, rather than a carrier protein, is involved. Owing to their positive charges, polyamines can bind the negative charge of phospholipids (PL), such as phosphatidic acid, phosphatidylserine, phosphatidylinositol, etc., and the affinity of this binding is greater for Sp than for the other amines [29]. However, elements other than a simple polyelectrolyte effect, which can modulate these interactions and make Sp a positive effector of CDDP uptake and Spd an ineffective molecule, should be considered. Regarding the direct complexing of polyamines by acidic groups of PL in membranes, it has been suggested [13] that polyamines can stiffen the membrane surface, clustering the acidic PL, and that Sp in particular might bridge PL domains and/or integral proteins and PL binding sites. This action on membranes could promote the conditions of increasing passive membrane permeability and thus CDDP accumulation. The lack of any selective action of Sp on the membrane of these sensitive and resistant cell lines could probably be explained by the fact that cisplatin-resistant 2008 cells, despite some changes in the cellular phospholipid composition, have the same membrane fluidity as sensitive cells [30].

The exact mechanism by which Sp inhibits the colony formation of our cell lines is not known. Some authors have reported that Sp might reduce the glutathione (GSH) pool [24] or itself exert direct toxic effects on cells without changing into active metabolites [12], thus affecting cellular functions, e.g. impairing mitochondrial activity [11]. This hypothesis is not tenable in our case, since C13* cells, which have 2-fold elevated GSH levels [14] and exhibit mitochondrial abnormalities [31] compared to 2008 cells, are as sensitive as parental cells to Sp. It has also been proposed that Sp might interfere with the binding of mRNA to ribosomes inhibiting protein synthesis, perhaps by competing with other cations for binding sites on the ribosomes, thus leading to ribosome dissociation [11]. Whatever its mechanism may be, Sp uptake alters the intracellular polyamine profile in our cells, mainly enhancing the intracellular Spd depletion. The exact consequences of this modified intracellular polyamine profile are not fully understood; the possibility that this condition may be a step of the chain of events that cause cells to be more sensitive to CDDP during colony formation cannot be excluded.

In conclusion, the results indicate that polyamine metabolism is not involved in the development of resistance since CDDP-sensitive and -resistant cell lines have similar polyamine content and similar sensitivity to exogenous Sp. However, exposure to micromolar concentrations of Sp can sensitise cells, even resistant ones, to CDDP owing, at least in part, to an increase in its accumulation following modulation of cell-membrane permeability.

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