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Preclinical paper

Combination of cisplatin–procaine complex DPR with anticancer drugs increases cytotoxicity against ovarian cancer cell lines

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DPR, *cis*-diamminechloro-[2-(diethylamino)ethyl 4-aminobenzoate, *N*4]-chloridoplatinum(II) monohydrochloride monohydrate, is a newly developed water-soluble platinum compound which posses minimal cross-resistance to cisplatin and shows relatively less side effects. In an attempt to establish whether the combination of DPR with other conventional anticancer drugs would be of any benefit we assessed *in vitro* the cytotoxic effects of combinations of DPR with the antimetabolites 5-fluorouracil (5-FU) and methotrexate (MTX), the alkylating agents mitomycin C (MMC) and cisplatin, the antimicrotubule agent taxol (TAX), and the intercalating agent of the anthracycline group doxorubicin (DOX) on murine M5076 ovarian reticulosarcoma and human A2780 ovarian carcinoma cells. These agents were selected because of their common use in the clinic and because they represent four distinct categories of antineoplastic mechanisms. Cells were incubated for 72 h in the presence of single or combined drugs, and the cytotoxic effect was determined by the MTT assay. The analysis of combination treatment was made by the isobole method. In human A2780 cells, an overall synergy was found for DPR combined with 5-FU, DOX and cisplatin. Synergistic effects were also observed for most combinations with MTX or MMC. A DPR concentration-dependent additivity and antagonism was seen at the highest MTX concentration (1 μM), while additive effects were observed for the combined treatments of DPR and low concentrations of MMC (0.008 and 0.0016 μM). Additive effects were also observed for the association of DPR and TAX over most combinations tested. In murine M5076 cells, synergism was the prevailing result observed when DPR was combined with 5-FU, DOX, MMC or cisplatin. When administered together with MTX we observed additivity over most combinations tested. These findings suggest that DPR, when simultaneously administered with standard anticancer agents, may be advantagious for cytokilling. [© 1998 Lippincott-Raven Publishers.]

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Introduction

Cis-diamminechloro-[2-(diethylamino)ethyl 4-aminobenzoate, *N*4]-chloridoplatinum(II) monohydrochloride monohydrate (DPR) is a newly developed highly water-soluble platinum triamine complex containing the local anesthetic procaine as ligand.[1] In previous studies[1,2] DPR showed good *in vitro* cytotoxic and *in vivo* antitumor activity, and appeared to overcome the *in vitro* resistance to cisplatin.[3] This latter effect may depend on its greater ability to bind DNA and accumulate in the cells.[3] In comparison with cisplatin, DPR appears to have less side effects, in particular nephrotoxicity[1,4] and neurotoxicity (as determined *in vitro* by the use of chick dorsal root ganglia, manuscript in preparation). By contrast, the two platinum drugs have a similar effect on the cell cycle and on the induction of apoptosis at the same molar concentration and time exposure.[5] Overall these data suggest that DPR may be useful in multidrug therapy.

The aim of the present study was to investigate the effects *in vitro* of this promising new platinum compound in combination with commonly used anticancer agents representing four distinct categories of antineoplastic mechanisms. The MTT assay was used to evaluate the cytotoxicity of the combinations of DPR with 5-fluorouracil (5-FU), methotrexate (MTX), cisplatin, mitomycin C (MMC), doxorubicin (DOX) and taxol (TAX) on murine M5076 ovarian reticulosarcoma and human A2780 ovarian carcinoma cells. The effects of the combined treatment were analyzed by the isobole method.

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Materials and methods

Chemicals

DPR was synthesised as previously described,[1] cisplatin was purchased from Sigma (St Louis, MO), 5-FU was provided by Roche (Milano, Italy), MMC was purchased from Kyowa Italiana Farmaceutici (Milano, Italy), DOX was purchased from Pharmacia and Upjohn (Milano, Italy) and MTX was provided by Lederle (Cyanamid Italia, Catania, Italy). TAX, obtained from Bristol-Myers Squibb (Sermoneta, Italy), was used starting from its clinical formulation.

Tumor cell lines

Human A2780 ovarian carcinoma cells were maintained in exponential growth in RPMI 1640 medium containing glutamine (2 mmol/l), gentamycin (100 μg/ml) and 10% fetal calf serum. Murine M5076 ovarian reticulosarcoma cells were maintained in culture in RPMI 1640 medium containing glutamine (2 mmol/l), gentamycin (100 μg/ml), sodium pyruvate (100 μg/ml) and 15% heat-inactivated horse serum.

In vitro studies

The combined effect of DPR and the various antineoplastic drugs was tested *in vitro* using a continuous exposure to both compounds for 72 h. Cells were plated in flat-bottomed microtiter plates at 1500/well (A2780) or 5000/well (M5076) and treated with single or combined agents. DPR was dissolved in distilled water (final range concentrations: M5076, 0.13-4.15 μM; A2780, 0.065-1.04 μM), DOX was dissolved in normal saline (final range concentrations: M5076 and A2780, 0.0164-164 nM), 5-FU was diluted in normal saline (final range concentrations: M5076, 0.156-2.5 μM; A2780, 1.25-20 μM), MTX was diluted in normal saline (final range concentrations: M5076, 0.4-6.4 μM; A2780, 0.0001-1 μM), MMC was dissolved in normal saline (final range concentrations: M5076, 0.025-0.4 μM; A2780, 0.0016-1 μM), cisplatin was diluted in normal saline (final range concentrations: M5076 and A2780, 0.065-1.04 μM), TAX was dissolved in normal saline (final range concentrations for A2780 cells, 0.625-10 nM). The final volume of each well was 200 μl. After 3 days an aliquot of 50 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) solution (2 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C. At the end of the culture period, microplates were centrifuged at 275 *g* for 5 min. Then, culture medium was carefully aspirated and 100 μl of 100% dimethylsulfoxide was added. Complete and homogeneous solubilization of formazan crystals was achieved after 10 min of incubation and vigorous shaking of well contents with a multichannel pipette. The absorbance was measured on a microculture plate reader 400 ATC (SLT, Vienna, Austria) at 540 nm.[6] IC_{50}s were calculated on the basis of probit analysis of single dose-response curves.

Data analysis

The analysis of combination treatment was made by the isobole method.[7] For a combination of compounds *A* and *B*, the combination index *D* is calculated by the equation:

$$\frac{A_c}{B_e} + \frac{B_c}{B_e} = D$$

where A_c and B_c are the concentrations of the compounds in the combination, and A_e and B_e are the concentrations of compounds which alone gave the same magnitude of effect.[8] If $D<1$, the effect of the combination was considered synergistic; if $D=1$, the effect was considered simply additive; if $D>1$, the effect was considered antagonistic. Each experiment was repeated at least six times to allow the calculation of *p* values using Student's *t*-test. Experimental *D* values for additivity (sham combinations) were calculated using combinations of two serial dilutions of DPR and of each anticancer agent used in association.

Results

Cytotoxic activity of single antineoplastic agents on A2780 and M5076 cells

Initially, cell lines were incubated with single agents in order to determine the range of concentrations to use in combination experiments. On M5076 cells the mean IC_{50}s ± SD were 99 ± 21 nM, 1.37 ± 0.44 μM, 9.3 ± 2.6 nM, 1.29 ± 0.35 μM, 0.48 ± 0.11 μM and 1.94 ± 0.47 μM for MMC, MTX, DOX, 5-FU, cisplatin and DPR, respectively. For A2780 cells the mean IC_{50}s ± SD were 51 ± 9 nM, 16 ± 9 nM, 14.7 ± 4.6 nM, 5.63 ± 0.32 μM, 0.49 ± 0.11 μM, 3.4 ± 0.3 nM and 0.48 ± 0.13 μM for MMC, MTX, DOX, 5-FU, cisplatin, TAX and DPR, respectively.

Combination of DPR and 5-FU

When DPR was associated with the antimetabolite 5-FU an overall significant synergism ($p<0.02$) was observed for human A2780 cells (Figure 1). Synergism was also evident in most analyzed drug associations on murine M5076 cells ($p<0.05$), even though we found additivity in some cases. In particular, additivity was observed for the combination of 2.5 μM 5-FU with 0.52–4.15 μM DPR and for the combinations of 0.26–1.04 μM DPR with 0.156 and 0.312 μM 5-FU (Figure 1).

Combination of DPR and MMC

On human A2780 ovarian carcinoma cells the association of DPR and the alkylating agent MMC resulted in a significant synergism ($p<0.02$) only when MMC was used at the highest concentrations (0.04–1 μM). At its lowest concentrations (0.0016 and 0.008 μM) additivity was observed (Figure 2). While the MMC concentrations seem to be important for the expression of synergism, no differences in terms of synergism were observed in relation to DPR concentrations. On murine M5076 ovarian reticulosarcoma cells the combination of DPR and MMC gave synergism in most cases ($p<0.05$). Differently from the human A2780 cell line, both the concentrations of MMC and DPR seem to influence the nature of the combined drug effect on murine M5076 cells. In fact, additivity was generally observed with combinations of high and low concentrations of both compounds, with only two exceptions, a low degree of antagonism (D=1.33, $p<0.01$) at the combination of 4.15 μM DPR with 0.4 μM MMC and synergism at the combination of 0.26 μM DPR with 0.025 μM MMC ($p<0.02$) (Figure 2).

Combination of DPR and DOX

On human A2780 cells the association between DPR and the antibiotic DOX gave a great synergism in all the tested combinations ($p<0.05$) (Figure 3). The exposure of murine M5076 cells to both DPR and DOX generally resulted in a significant synergism ($p<0.05$) only when each drug was combined at its highest concentrations with all the concentrations of the second compound, with the exception of the combination of 164 nM DOX with 4.15 μM DPR, where we found additivity. Conversely, additivity was mainly observed when the lower concentrations of DPR and DOX were combined, with the exception of the combinations of 0.164 and 0.0164 nM DOX with 0.26 μM DPR, where synergism was again significant ($p<0.05$) (Figure 3).

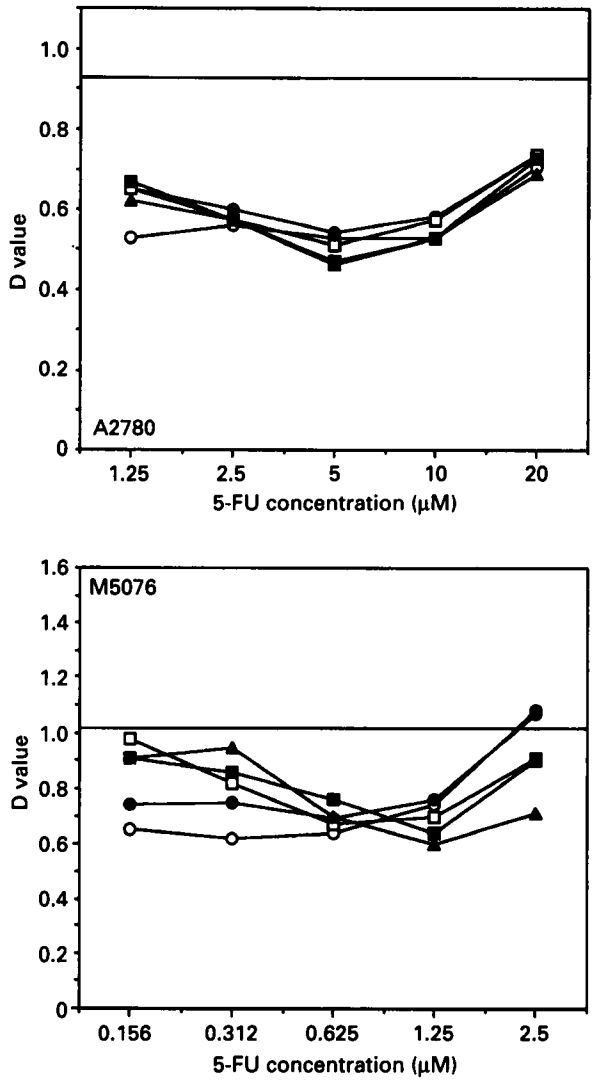

Figure 1. *D* values obtained for the combined treatment of A2780 and M5076 cells with DPR and 5-FU. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and 5-FU [A2780, *D* value for DPR=0.99±0.21 (SD); *D* value for 5-FU=0.82±0.17 (SD); mean *D* value=0.93±0.21 (SD); M5076, *D* value for DPR=1.02±0.20 (SD); *D* value for 5-FU=1.01±0.31 (SD); mean *D* value=1.02±0.28 (SD)]. DPR concentrations: A2780, 1.04 μM (○), 0.52 μM (●), 0.26 μM (□), 0.13 μM (■) and 0.065 μM (▲); M5076, 4.15 μM (○), 2.07 μM (●), 1.04 μM (□), 0.52 μM (■) and 0.26 μM (▲). For more clarity bars, representing SD, were omitted.

Combination of DPR and MTX

When human A2780 cells were tested with the combination of DPR and the analog of folic acid MTX, synergism was found for most drug combinations ($p < 0.02$). Only when MTX was utilized at the concentration of 1 μM did we observe antagonism, for the combination with 0.26-1.04 μM DPR ($p < 0.001$), and additivity, for the combination with 0.065 and 0.13 μM DPR (Figure 4). On murine M5076 cells additivity was found for most drug combinations with a few exceptions. In fact, antagonism was observed with the combinations of 6.4 μM MTX with 2.07 and 4.15 μM DPR, 0.8 μM MTX with 1.04 μM DPR and, finally, 0.4 μM MTX with 0.26-1.04 μM DPR ($p < 0.05$). Synergism was observed when the lowest concentration of DPR (0.26 μM) was combined with 1.6-6.4 μM MTX ($p < 0.01$) (Figure 4).

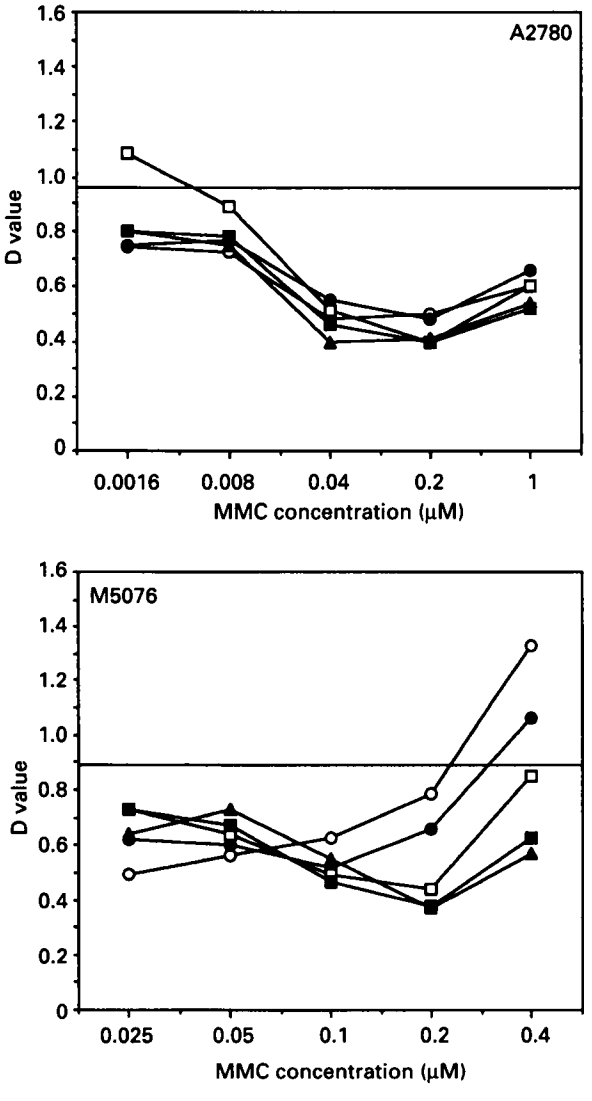

Figure 2. *D* values calculated for the combined treatment with DPR and MMC. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and MMC [A2780, *D* value for MMC=0.90±0.56 (SD); mean *D* value=0.96±0.35 (SD); M5076, *D* value for MMC=0.75±0.11 (SD); mean *D* value=0.89±0.21 (SD)]. Experimental *D* values of sham combinations and the concentrations of DPR are reported in the legend of Figure 1.

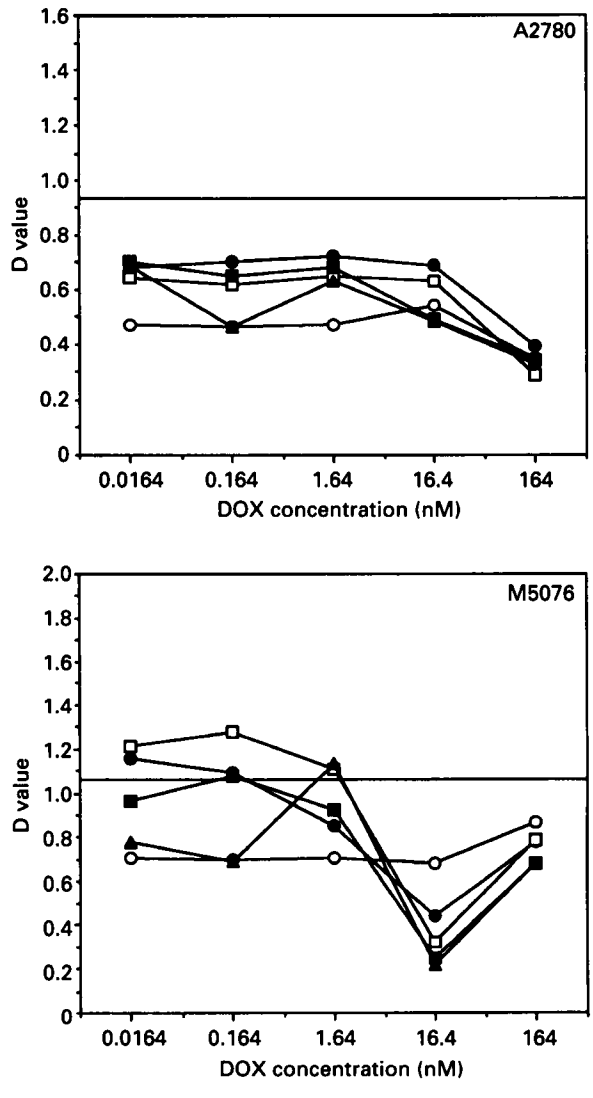

Figure 3. *D* values obtained for the combined treatment of A2780 and M5076 cells with DPR and DOX. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and DOX [A2780, *D* value for DOX=0.80±0.28 (SD); mean *D* value=0.93±0.25 (SD); M5076, *D* value for DOX=1.10±0.22 (SD); mean *D* value=1.06±0.21 (SD)]. Experimental *D* values of sham combinations and the concentrations of DPR are reported in the legend of Figure 1.

Combination of DPR and cisplatin

On human A2780 cells the combined treatment with cisplatin and DPR resulted in a significant synergism ($p < 0.01$) in all the drug combinations tested (Figure 5). When murine M5076 cells were incubated with the association of cisplatin and DPR a significant ($p < 0.05$) cellular synergism was evident in all combinations but four. In fact, additivity was observed at the combinations of 1.04 μM cisplatin with 1.04 and 2.07 μM DPR and 0.13 or 0.065 μM cisplatin with 1.04 μM DPR (Figure 5).

Combination of DPR and TAX

The combined treatment with DPR and the antimicrotubule agent TAX was tested on human A2780 ovarian

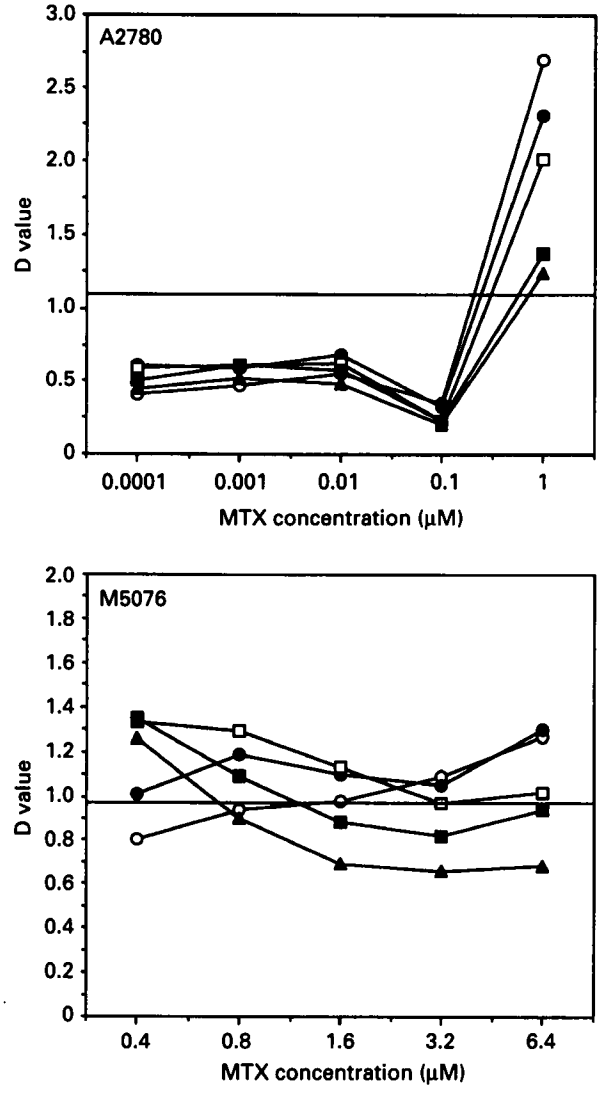

Figure 4. Representation of *D* values in the combined experiments involving DPR and MTX. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and MTX [A2780, *D* value for MTX=1.28±0.48 (SD); mean *D* value=1.10±0.35 (SD); M5076, *D* value for MTX=0.94±0.27 (SD); mean *D* value=0.97±0.25 (SD)]. Experimental *D* values of sham combinations and the concentrations of DPR are reported in the legend of Figure 1.

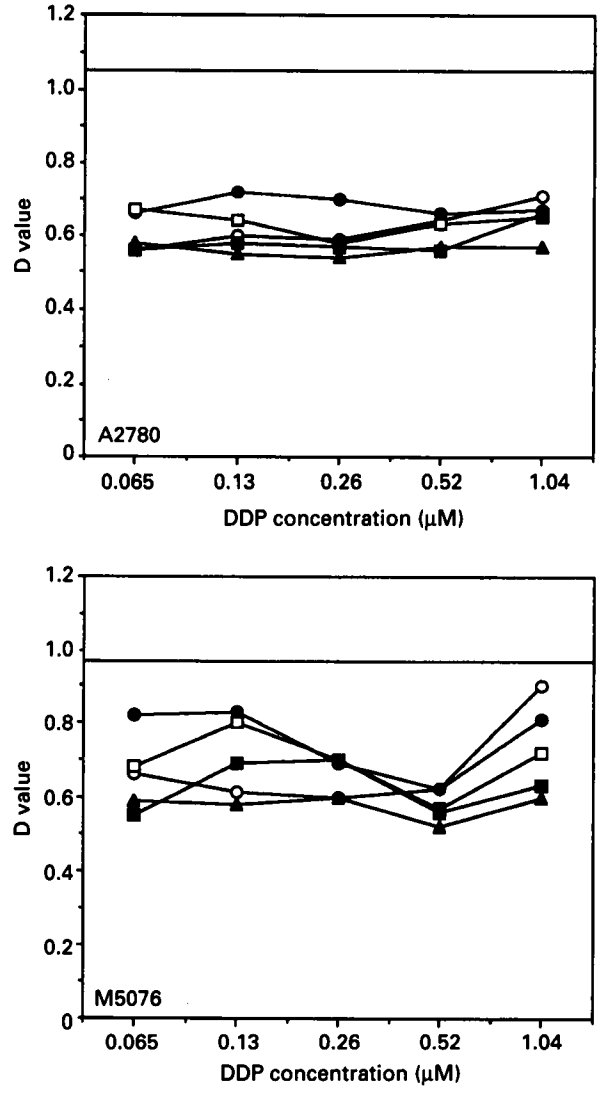

Figure 5. *D* values calculated for the combined treatment with DPR and cisplatin. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and cisplatin [A2780, *D* value for cisplatin=1.11±0.29 (SD); mean *D* value=1.05±0.26 (SD); M5076, *D* value for cisplatin=0.92±0.15 (SD); mean *D* value=0.97±0.18 (SD)]. DPR concentrations used on M5076 cells: 2.07 μM (○), 1.04 μM (●), 0.52 μM (■), 0.26 μM (□) and 0.13 μM (▲). Experimental *D* values of sham combinations for DPR and the concentrations of DPR used on A2780 cells are reported in Figure 1.

carcinoma cells. In this case the prevailing result of the combined treatment was additivity. Significant antagonism ($p<0.02$) and synergism ($p<0.05$) were observed only with a few drug combinations. In particular, antagonism was found when 10 nM TAX was combined with 0.26–1.04 μM DPR and when 0.625 nM TAX was associated with 0.065–0.26 μM DPR, while synergism was observed for the combination of 5 nM TAX with 0.065 and 0.13 μM DPR (Figure 6).

Discussion

Greater-than-additive (synergistic) cell killing was observed with many of the drug combinations examined in this study in both ovarian cancer cell lines. In general, the magnitude of the synergy was greater in the human than in the murine cell line. All drug combinations tested in this study contained DPR, a promising new cationic platinum-triamine complex, which contains procaine hydrochloride.[1] In previous work,[1,2] we have demonstrated that DPR at equimolar concentrations has a similar or even stronger cytotoxic activity than cisplatin. Furthermore, the combination of DPR and cisplatin showed a therapeutic advantage over single drug treatment in different tumor models[2] and has demonstrated promise at the preclinical level for its ability to circumvent the acquired resistance to cisplatin, both *in vitro* and *in vivo*.[3] Therefore, we hypothesized that DPR may have a role in multidrug therapy with cisplatin.[3]

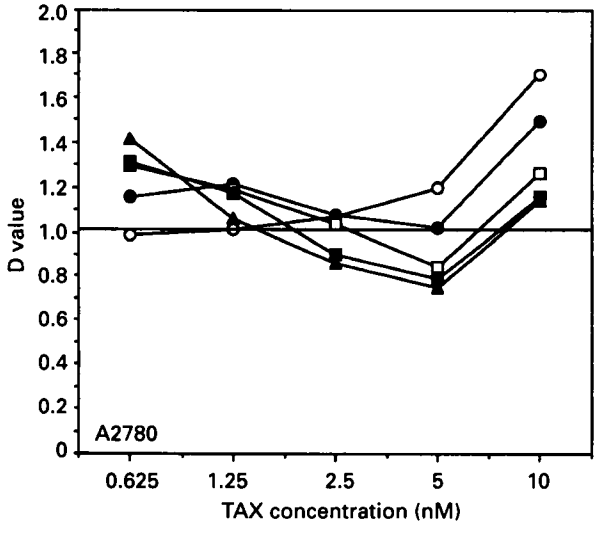

Figure 6. *D* values in the combined experiments involving DPR and TAX. The experimental mean *D* value for additivity was calculated using combinations of two serial dilutions of DPR and TAX on A2780 cells [*D* value for DPR=0.99±0.21 (SD); *D* value for TAX=1.05±0.27 (SD); mean *D* value=1.01±0.24 (SD)]. The concentrations of DPR are reported in the legend of figure 1.

Although the importance of cisplatin in combination chemotherapy against ovarian carcinoma has been well documented,[9–12] renal and neurologic toxicities limit the treatment efficacy with this platinum drug. It has previously shown that DPR has outstanding anticancer efficacy, no apparent nephrotoxic effect and a high water solubility.[1] Moreover, DPR induces a lower neurotoxic effect than cisplatin as determined by the use of chick dorsal root ganglia *in vitro* (manuscript in preparation). Overall these observations provided a rational basis for a preclinical screening of potentially valuable DPR combinations with other anticancer agents. Each anticancer drug utilized in this work represents an example of the main groups of chemotherapeutic agents, characterized by different mechanisms of action and used in the clinic for the treatment of tumors of different histologic origin (5-FU and MTX, antimetabolite agents; DOX, anthracycline, intercalating agent; MMC and cisplatin, alkylating agents; TAX, antimicrotubule agent).

Our results clearly show that synergism was mainly observed independently of the mechanism of action of the compounds tested with DPR. In fact, on human A2780 ovarian carcinoma cells DPR showed a significant and complete synergism when combined with the antimetabolite 5-FU, the anthracycline DOX and the alkylating agent cisplatin. Synergism was also expressed by most combinations of DPR and the alkylating agent MMC or the analog of folic acid MTX. Only when DPR was given in association with the antimicrotubule agent TAX was additivity the prevailing result.

Similar, although not identical, results were observed when murine M5076 ovarian reticulosarcoma cells were used as cellular target. In this case a complete synergism was never observed, although it remained the prevailing effect of the combinations of DPR with 5-FU, MMC, DOX and cisplatin.

The mechanisms underlying the synergistic effect of DPR and anticancer agents with different antineoplastic mechanisms on ovarian tumor cells are obscure. Transport into tumor cells may be a critical step of the action of most antitumor drugs. One possibility is, therefore, that when DPR is administered with anticancer compounds, one agent may somehow facilitate the uptake of the other. On the other hand, the experimental design emphasizes the end results of drug combinations rather than the mechanism of synergistic or antagonistic interactions and did not address the schedule-dependence of effects. We

should also point out that thus far only one human ovarian cancer cell line has been tested and that the results should be confirmed with more cell lines. Despite these limitations, it will be of interest to see whether the present results have any predictive value.

In conclusion, our findings show that DPR can appreciably enhance the activity of some drugs aganist murine and human test models. Clinically, the most promising appear to be the combinations of DPR with 5-FU, DOX, MMC and cisplatin. Experiments are in progress to elucidate the mechanism underlying the synergistic effects here observed.

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