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In vitro activity of titanocenedichloride versus cisplatin in four ovarian carcinoma cell lines evaluated by a microtiter plate ATP bioluminescence assay

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Titanocenedichloride (MKT 4) is a novel anticancer drug with a broad spectrum of activity in mammalian tumors. We investigated the anticancer efficacy of MKT 4 versus cisplatin and its chemomodulation by buthionine sulfoximine (BSO) in four different human ovarian carcinoma (OvCA) cell lines derived from both primary (A2780. OTN 14) and recurrent tumors (SKOV-3 and OV-MZ-1b) using an *in vitro* microplate ATP bioluminescence assay (ATP-TCA). Sensitivity against cisplatin was higher in A2780 and OTN 14 compared with MKT 4, whereas the opposite was found in SKOV-3 and OV-MZ-1b cells. In A2780, SKOV-3 and OV-MZ-1b, the cytotoxicity of both agents could be effectively improved by BSO with supraadditive effects observed for MKT 4 in all three cell lines. In OTN 14, however, BSO treatment failed to increase the cytotoxicity of both cisplatin and MKT 4. These results suggest antineoplastic activity of MKT 4 in cisplatin-sensitive and mainly in cisplatin-resistant OvCA cells which can be significantly modulated by BSO-mediated glutathione depletion. Since antineoplastic activity of both cisplatin and MKT-4 observed in OTN 14 could not be reversed by BSO, other mechanisms of drug resistance different from the glutathione redox cycle are likely to be important for both metal compounds.

Key words: ATP bioluminescence assay, buthionine sulfoximine, cisplatin, ovarian carcinoma, titanocenedichloride.

Introduction

Since the late 1960s, when the antitumor activity of cisplatin (DDP) was discovered accidentally during an electrobiological experiment,[1] a growing body of interest in the development of cytotoxic metal compounds has arisen.[2,3] During the last two decades, the antineoplastic properties of a variety of inorganic or metallorganic molecules have been studied. The central atom of these substances can be recruited from a broad range of early and late transitional metals such as titanium, vanadium, niobium, iron, ruthenium, rhodium, copper, gold and platinum, or main group metals like tin, germanium or gallium.[3,4] Among non-platinum agents, gallium nitrate (NSC-15200),[5] spirogermanium (NSC-192965),[6] budotitane (BDT)[7] and titanocenedichloride (MKT 4)[8] are under clinical phase I or phase II investigation.[2,3]

MKT 4 is an early transitional metal complex with antineoplastic activity against a variety of mammalian neoplasms including both cell lines and native tumors.[4–14] In animals treated with MKT 4, a 10-fold reduced toxicity compared to cisplatin could be observed.[12] In human tumors grown *in vitro* or heterotransplanted to nude mice, the anticancer efficacy of MKT 4 was similar or even better compared to cisplatin when administered at equitoxic dosages.[4,15] Additionally, MKT 4 provided a favorable therapeutic index with reduced nephrotoxicity and neglectable myelotoxicity.[16,17] The major adverse effects of MKT 4 observed in mice were a remarkable but mainly transient liver toxicity and metabolic disturbances resulting in hypoglycemic episodes lasting over several hours.[16,18]

In studies using ovarian carcinoma (OvCA) cell lines which were selected for resistance against cisplatin or doxorubicin (DOX) by stepwise exposure of the sensitive parental line to increasing concentrations of the particular drug, a lack of cross-

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resistance between MKT 4 and both cisplatin and DOX could be observed.[12] Activity of both metal complexes could be improved by glutathione (GSH)-depletion mediated by 30 μM buthionine sulfoximine (BSO) with highest efficacy in drug-resistant cell lines.[12] Therefore the GSH redox cycle which has been found to play an important role in cisplatin-resistance,[19] seems to contribute also to resistance against MKT 4.

Drug resistance generated in the laboratory, however, might be somewhat different to that which occurs under clinical conditions. Therefore, this trial was initiated to investigate the anticancer activity of MKT 4 versus cisplatin and its chemomodulation by BSO in OvCA cell lines derived from both untreated patients and those whose tumors exhibited features of intrinsic or acquired platinum resistance.

Measurement of ATP bioluminescence utilizing the luciferin–luciferase reaction has been proven to be a sensitive and valid method to assess the cytotoxicity of anticancer agents in human tumor cells *in vitro*.[20] Showing similar accuracy, the sensitivity of ATP-based assays was found to be 10-fold higher compared with the MTT assay.[21] Both assays have been used for pretherapeutic drug screening, providing potential applications in clinical oncology.[22–24] Recently, a microtiter plate ATP tumor chemosensitivity assay (ATP-TCA) has been developed utilizing a simplified kit technique.[25] The ATP-TCA seems to be a valuable method to test the chemosensitivity of both tumor cell lines[26] and clinical tumors,[13,14,25,27,28] and is thus regarded as a suitable method to evaluate the cytotoxicity of MKT 4 versus cisplatin and the modulating effect of BSO in human OvCA cell lines.

Materials and methods

Ovarian carcinoma cell lines

In the experiments described herein, four different OvCA cell lines derived from both untreated patients and from those which had been exposed previously to a variety of cytotoxic agents and/or irradiation were studied. The individual characteristics of all cell lines are summarized in Table 1. SKOV-3[29] was purchased from the tumor cell bank, Deutsches Krebsforschungszentrum (Heidelberg, Germany). A2780[30] was obtained from TC Hamilton (Fox Chase Cancer Center, Philadelphia, PA). OV-MZ-1b[31] was a kind gift from V Möbus (Department of Obstetrics and Gynecology, University of Ulm, Germany) and OTN 14[32] was generously provided by LG Poels (Department of Pathology, University of Nijmegen, The Netherlands). Cell lines were maintained as monolayer cultures in cell culture medium (CCM), RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 μg/ml insulin, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C, in a humidified 95% air/5% CO_2 atmosphere (all media and supplements from Gibco/BRL, Paisley, UK). Cultures were passaged twice a week at a 1:2 ratio. Periodical checks for mycoplasma contamination gave negative results (33528 dye; Hoechst, Frankfurt, Germany).

Drugs

A commercial formulation of cisplatin (Platinex®; Bristol-Myers Squibb, Munich, Germany) was used

Table 1. Characteristics of four ovarian carcinoma cell lines tested with the ATP-TCA

Cell line	Histology	Origin of sample	Therapy			
			Pre-biopsy		Post-biopsy	
			Treatment	Response	Treatment	Response
A2780	serous adenocarcinoma	tumor	none	NK	NK	NK
OTN 14	mucinous adenocarcinoma	ascites	none	NK	NK	NK
SKOV-3	adenocarcinoma	ascites	thiotepa	NK	NK	NK
OV-MZ-1b	adenocarcinoma	ascites	2 × CPA, 13 × CAP, 3 × CP, irradiation (15 Gy)	PD	radio-yttrium i.p. (40 μCi)	PD died

Abbreviations: NK, not known; PD, progressive disease; CPA, cyclophosphamide; CAP, CPA + doxorubicin + cisplatin; CP, CPA + cisplatin, i.p. intraperitoneal instillation.

for this study. Preparation and storage of stock solutions was performed according to the instruction of the manufacturer. MKT 4 (lyophilisate) was kindly provided by Medac (Hamburg, Germany). MKT 4 was dissolved freshly for each assay using an appropriate amount of CCM. L-BSO was purchased from Sigma (St Louis, MO). A stock solution which was cryopreserved at −18°C until usage was prepared dissolving the powdered drug in CCM.

Both cytostatics were tested at six different test drug concentrations (TDC) which were prepared directly on the plates by serial 50% dilutions. The tested dose-range for cisplatin was 200–7600 ng/ml with a referential concentration (i.e. 100% TDC) of 3800 ng/ml according to the clinical peak plasma concentration (PPC) after i.v. administration of a standard dosage.[33] Currently, only preliminary data exist about the pharmacokinetics of MKT 4 in humans. With respect to its 10-fold reduced toxicity compared with cisplatin,[12] a concentration of 37 500 ng/ml was considered as an adequate approach for 100% TDC. Thus the studied dosages ranged between 2300 and 75 000 ng/ml. Cisplatin and MKT 4 were tested as single agents and in combination with BSO at a fixed concentration of 25 μM, which was found to exhibit only a marginal antineoplastic activity producing a 10–20% reduction of cell growth in all OvCA cell lines tested (data not shown).

Chemosensitivity assay

For assessment of the *in vitro* activity of cisplatin and MKT 4, a microtiter plate ATP-TCA which now is available as a test kit (TCA-100®; DCS, Hamburg, Germany) was used.[25,28] The original ATP-TCA methodology, which was primarily developed for culturing native tumor cells, was slightly modified for the use of permanent cell lines according to the proposals of Andreotti *et al.*[26]

Briefly, CCM as described above was used instead of the selective Complete Assay Medium (CAM) which is provided by the TCA-100® kits. Furthermore the original round-bottom 96-well polypropylene microtiter plates were exchanged by 96-well flat-bottom polystyrene plates (Falcon; Becton Dickinson, Heidelberg, Germany). The other assay materials were provided by the TCA-100® kits if not stated separately.

Tumor cells from sub-confluent monolayers were harvested by trypsinization (0.02% NaEDTA in 0.05% trypsin; Boehringer, Mannheim, Germany). After washing in Hank's balanced salt solution (HBSS; Gibco/BRL) and centrifugation (5 min at 100 *g*), cells were resuspended in CCM and aspirated several times using sterile 16-gauge needles and 1 ml syringes (Braun, Melsungen, Germany) in order to disperse cell aggregates. Viability of the resultant single cell suspensions were determined by Trypan blue dye exclusion (0.2%; Merck, Darmstadt, Germany) and phase contrast microscopy. Suspensions then were adjusted to a final concentration of 1×10^4 viable cells/ml by adding an appropriate amount of CCM. After preparing the above mentioned TDCs, cells were seeded into the culture plates at 1000 cells/well (i.e. 5×10^3 cells/ml). Each TDC was tested in triplicates. Two controls, one with maximum ATP inhibitor (maximum inhibition, MI) and one with CCM (no inhibition, M0), were added to six wells of each culture plate. All experiments were repeated twice. Cultures were incubated at 37°C, 95% air/5% CO_2 and 95% humidity.

After 6 days of incubation, cellular ATP was extracted by pipetting 50 μl of tumor cell extraction reagent (TCER) into each well of the culture plates. ATP measurements were performed utilizing a LB 953-luminometer (Berthold, Wildbad, Germany) after automatically mixing a 50 μl aliquot of each cell lysate with 55 μl luciferin–luciferase light reagent (Lu-Lu). The counting time was 10 s with a 4 s delay. Results were expressed as relative light units (RLU = photons/10). The survival fraction for each drug concentration (SF_{Test}) was determined as:

$$SF_{Test} = (RLU_{Test} - RLU_{MI}/(RLU_{M0} - RLU_{MI})$$

Data then were graphed, resulting in individual survival curves for each cell line and drug, respectively. The concentrations producing a 50% reduction of SF versus control (IC_{50}) were calculated by non-linear regression analyses fitting to a sigmoidal curve (Prism® software; GraphPAD, San Diego, CA). The activities of both agents and the influence of BSO on their activities expressed in the same OvCA cell line were analyzed by non-paired student's *t*-tests with $p \leq 0.05$ indicating statistical significance. In order to analyze the effect of BSO on the cytotoxicity of cisplatin and MKT 4, respectively, the true survival curves for the combinations were compared with a theoretical one. Assuming both an above mentioned average SF reduction versus control of 15% which was produced by 25 μM BSO and additive drug effects of BSO and either cisplatin or MKT 4 the theoretical curves were calculated as:

$$SF_{Comb} = SF_{Drug} \times 85\%[\text{SF versus control for BSO}]$$

Results

IC_{50} values for cisplatin and MKT 4 are shown in Table 2. With respect to its 10-fold reduced *in vivo* toxicity compared with cisplatin, MKT 4 was found nearly equipotent to cisplatin in A2780 cells. The novel drug produced significant less cytotoxicity than cisplatin in OTN 14 cells. In cell lines derived from pretreated patients, however, MKT 4 showed greater antineoplastic efficacy with a maximum effect in OV-MZ-1b which was the cell line exhibiting the highest resistance to cisplatin *in vitro* and *in vivo*.[31]

Co-incubation with 25 μM BSO influenced the cytotoxicity of both cisplatin and MKT 4 with marked differences depending on the cell line tested as shown in Figure 1. Results are presented as IC_{50} values in percent TDC in order to facilitate the comparison between cisplatin and MKT 4. Among the cell lines tested, OTN 14 was almost non-responsive to GSH depletion, since BSO improved the IC_{50} for MKT 4 by 1.2-fold and even deteriorated the IC_{50} for cisplatin by 1.1-fold. In contrast, OV-MZ-1b and A2780 cells exhibited sensitivity to treatment with BSO. The IC_{50} values for cisplatin were improved by 1.4 (OV-MZ-1b) and 3.8 (A2780) fold, respectively. The IC_{50} values for MKT 4 were even more effectively lowered by BSO showing a 3.5-fold decrease in OV-MZ-1b and a 7.4-fold decrease in A2780. SKOV-3 exhibited intermediate sensitivity to BSO with an 1.8-fold improvement of IC_{50} for cisplatin and an 2.2-fold improvement for MKT 4. Simultaneous *in vitro* treatment with BSO reduced the IC_{50} for cisplatin in SKOV-3 and OV-MZ-1b cells, reaching levels as low as observed for cisplatin alone in A2780 or OTN 14, respectively. Moreover, addition of BSO produced a reduction of IC_{50} for MKT 4 in A2780 and SKOV-3 cells reaching levels similar or even lower than those observed for OV-MZ-1b, which expressed the highest *in vitro* sensitivity to the single agent.

More detailed information is given in Figures 2–5 showing survival curves for single agent cisplatin (Figures 2–5, top) and MKT 4 (Figures 2–5, bottom), respectively, and the combinations with BSO. For the cisplatin/BSO combination additive effects were found in SKOV-3 and OV-MZ-1b cells, whereas a subadditive or even antagonistic action of the com-

Table 2. Comparison between IC_{50} values for cisplatin and MKT 4 in four ovarian carcinoma cell lines *in vitro* evaluated by the ATP-TCA

Cell line	Cisplatin (ng/ml)	SD	MKT 4 (ng/ml)	SD	MKT 4/cisplatin
A2780	946.2	41.8	13275.5	1221.0	14.0
OTN 14	1166.7	12.2	37087.5	3637.5	31.8
SKOV-3	1812.6	22.8	14962.5	2775.0	8.3
OV-MZ-1b	2872.8	68.4	6300.0	1237.5	2.2

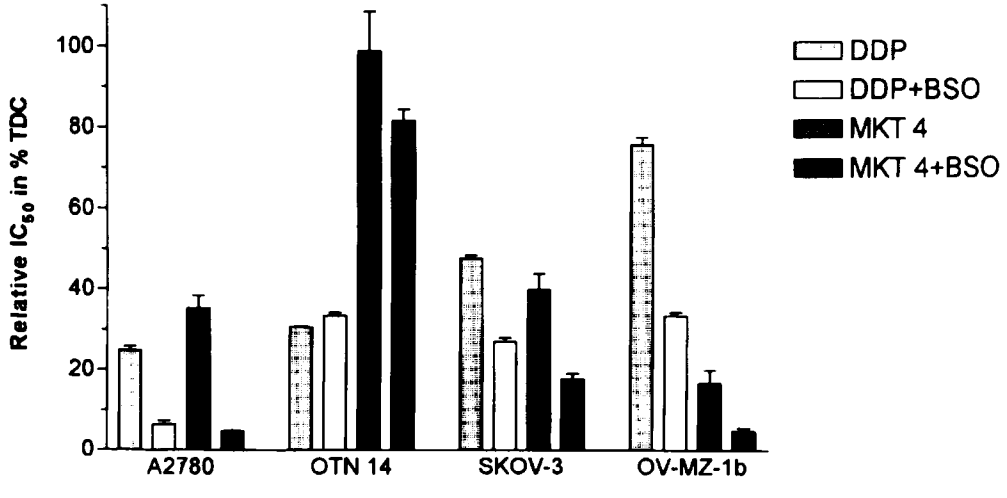

Figure 1. IC_{50} values for cisplatin (DDP) and MKT 4 alone and in combination with 25 μM BSO. Concentrations are expressed as percent TDC. Error bars represent standard deviations.

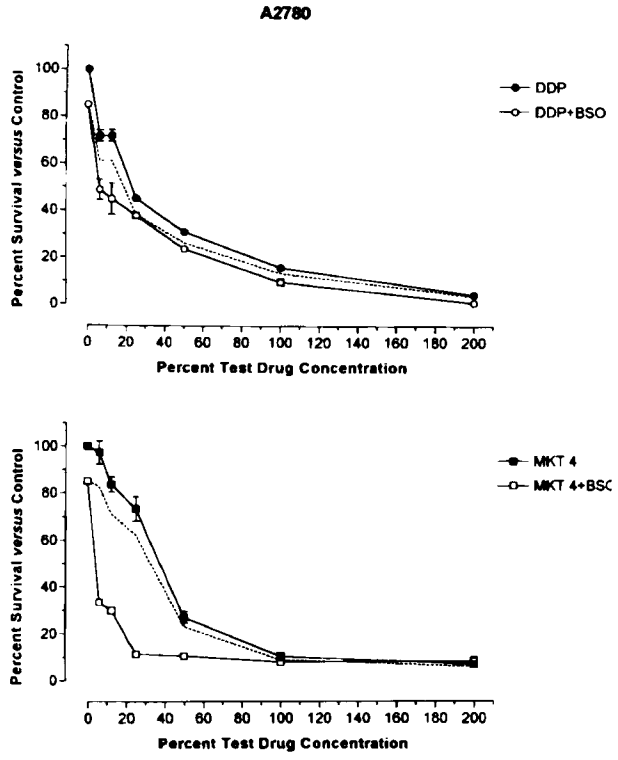

Figure 2. Survival of A2780 ovarian carcinoma cells exposed to increasing concentrations of cisplatin (DDP; top) or MKT 4 (bottom) alone and in combination with 25 μM BSO. Concentrations are expressed as percent TDC. Error bars represent standard deviations. The dashed lines show the theoretically expected survival if BSO and either cisplatin or MKT 4 produced additive cytotoxicity.

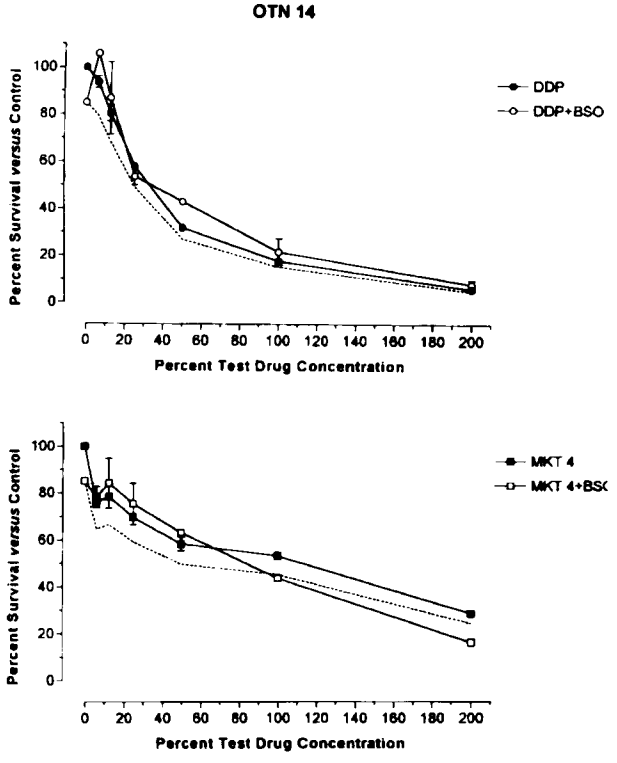

Figure 3. Survival of OTN 14 ovarian carcinoma cells exposed to increasing concentrations of cisplatin (DDP; top) or MKT 4 (bottom) alone and in combination with 25 μM BSO. Concentrations are expressed as percent TDC. Errors bars represent standard deviations. The dashed lines show the theoretically expected survival if BSO and either cisplatin or MKT 4 produced additive cytotoxicity.

bination was found in OTN 14. In A2780, however, the combination effect was more likely to be supra-additive and most pronounced at the low concentration level between 6.25% TDC and 25% TDC.

A slightly different panel of effects was observed for the MKT 4/BSO combination. Like cisplatin/BSO, the combined activities were subadditive in OTN 14 and supra-additive in A2780. The major effect of BSO on the cytotoxicity of MKT 4 in the latter cell line could be found for lower concentrations between 6.25 and 50% TDC. An opposite effect could be seen in SKOV-3 cells. Supra-additive drug effects mainly occurred at higher concentrations above 50% TDC. In OV-MZ-1b cells, BSO potentiated all concentrations of MKT 4 with no marked difference between low and higher dosages.

Discussion

In this study, the *in vitro* activity of MKT 4 versus cisplatin in four different OvCA cell lines derived from both previously untreated patients and those relapsing after chemotherapy was evaluated using a microtiter plate ATP bioluminescence assay. Both cytostatics were studied as single agents and in combination with 25 μM BSO, which showed only a marginal antineoplastic effect. For both metal complexes remarkable intra- and interindividual differences in antineoplastic efficacy and modulation by BSO could be observed. Generally, results obtained for cisplatin seem to fit well to the clinical situation since cell lines derived from primary tumors exhibited a stronger sensitivity to cisplatin compared with cell lines from recurrent OvCAs. Additionally, cisplatin was found to be more effective compared to MKT 4 in primary tumors, whereas the opposite result could be observed in pretreated tumors, albeit only OV-MZ-1b was obtained from a cisplatin-pretreated patient (see Table 1).

As Harstrick *et al.* reported previously, A2780 cells proved to be sensitive to both cisplatin and MKT 4.[12] Controversely, OTN 14 which showed at least a moderate sensitivity to cisplatin, was resistant to MKT 4 with the highest IC_{50} value observed among all four cell lines. The opposite effect could

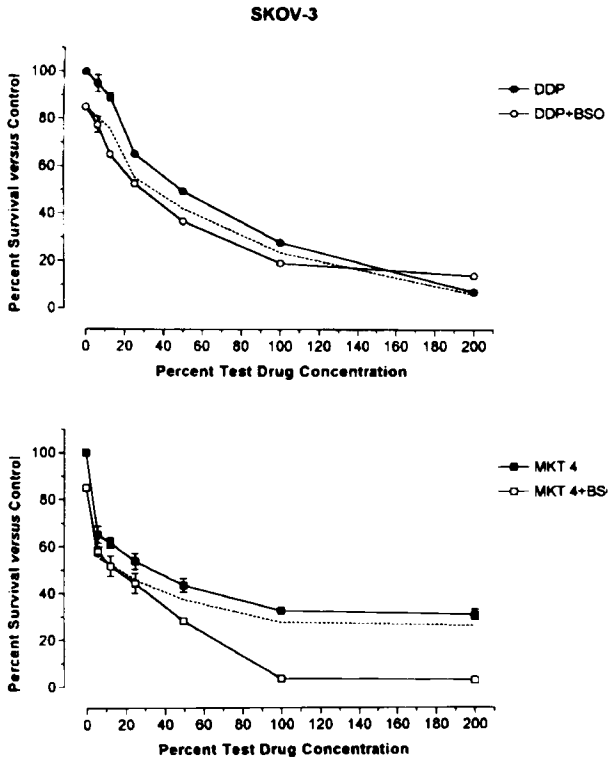

Figure 4. Survival of SKOV-3 ovarian carcinoma cells exposed to increasing concentrations of cisplatin (DDP; top) or MKT 4 (bottom) alone and in combination with 25 μM BSO. Concentrations are expressed as percent TDC. Error bars represent standard deviations. The dashed lines show the theoretically expected survival if BSO and either cisplatin or MKT 4 produced additive cytotoxicity.

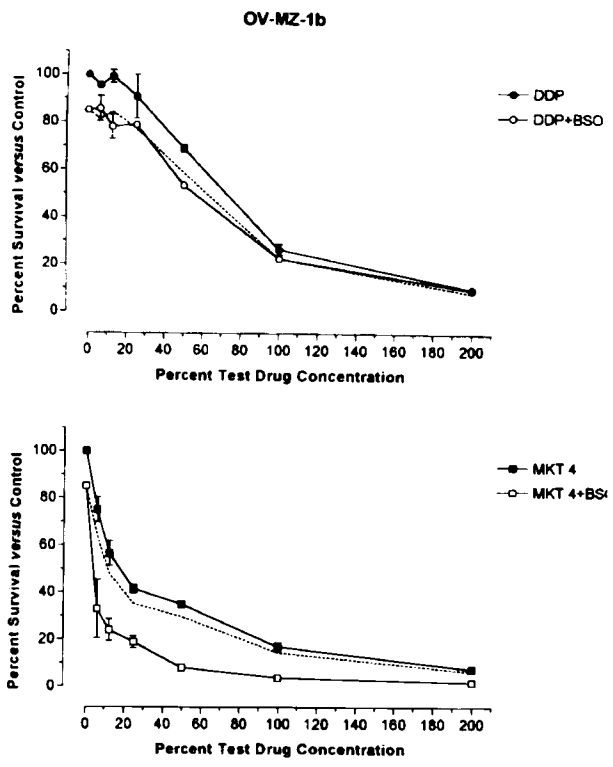

Figure 5. Survival of OV-MZ-1b ovarian carcinoma cells exposed to increasing concentrations of cisplatin (DDP; top) or MKT 4 (bottom) alone and in combination with 25 μM BSO. Concentrations are expressed as percent TDC. Error bars represent standard deviations. The dashed lines show the theoretically expected survival if BSO and either cisplatin or MKT 4 produced additive cytotoxicity.

be found for SKOV-3 and OV-MZ-1b, which were more resistant to cisplatin, but fairly or even strongly sensitive to MKT 4.

Studying A2780 cells and their cisplatin-resistant variants which were produced *in vitro* by exposing the sensitive parental line continuously to increasing concentrations of cisplatin, Harstrick *et al.* found a lack of cross-resistance between cisplatin and MKT 4.[12] In a former study with native OvCA cells obtained from clinical tumors, we were able to confirm these results in that a number of tumors showing *in vivo* and *in vitro* resistance to cisplatin were sensitive to MKT 4.[14] The proportion of tumors expressing resistance to both organometallic compounds however, was higher in the recurrent than in the primary group. This suggested that a proportion of clinical ovarian neoplasms are likely to exhibit cross-resistance between cisplatin and MKT 4.

Wild-type A2780 cells and their resistant variants have been extensively studied as a model testing the relevance of GSH and GSH-related enzymes for platinum resistance.[34] Clearly, this cell line seems to be rather sensitive to GSH depletion induced by BSO, showing a remarkable enhancement of sensitivity to platinum compounds,[19,35] alkylating agents,[19,36] irradiation[37] and MKT 4.[12] Accordingly, we were able to improve the sensitivity of A2780 cells to both cisplatin and MKT 4 effectively when BSO was added to the culture medium. BSO also induced an enhanced sensitivity of OV-MZ-1b and SKOV-3 against both metal complexes. In these two cell lines, the cisplatin/BSO combination was found to be additive whereas even supra-additivity was observed for MKT 4/BSO. Generally, MKT 4 could be more effectively potentiated by BSO than cisplatin. In contrast to results with other cell lines, little or no effect of BSO on the chemosensitivity of OTN 14 against both metal compounds could be found.

As mentioned above, the GSH redox cycle clearly plays an important role for the resistance of OvCA cells to platinum analogs, alkylating agents or irradiation. BSO-mediated cellular GSH depletion is able to enhance the toxicity of both cisplatin and MKT 4 in sensitive and resistant cells *in vitro* as

could be demonstrated by us and others.[12] Results found with OTN 14, however, must be taken into account before generalizing observations which were made using highly artificial model systems such as tumor cell lines with experimentally induced drug resistance. The activity of MKT 4 which has been observed in DOX-resistant variants of A2780 does not suggest MKT 4 as a substrate of P-glycoprotein.[12] Intranucleic mechanisms such as enhanced activity of repair enzymes are known to play an important role in platinum resistance by recovering drug-damaged DNA.[38,39] As recently demonstrated by Murray and Harding, immediate DNA complexation seems to determine the anticancer efficacy of biologically active metallocene dihalides such as titanocenedichloride.[40] One thus can presume that DNA repair mechanisms as described above are likely to influence the sensitivity of tumor cells against MKT 4. Experimental trials studying the modulation of both MKT 4 and cisplatin by different inhibitors of DNA repair are under way in our laboratory. These investigations, also including BSO, which was found to be potent modulator of cisplatin and MKT 4, should be extended to clinical tumor specimens in order to obtain more detailed informations about possible differences between intrinsic and induced resistance to anticancer metal complexes.

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