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Cisplatin-resistant bladder carcinoma cells: enhanced expression of metallothioneins

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Abstract Cisplatin is one of the most potent cytotoxic drugs and in chemotherapy has ameliorated numerous tumors. Nevertheless, resistance to cisplatin is a problem that is encountered in the chemotherapy of urologic tumors, especially transitional cell carcinomas. In order to improve definition of the mechanisms of cisplatin-resistance we established a series of cisplatin-resistant sublines from the cell line RT 112 in increasing concentrations of cisplatin. The most resistant subline CP3 is approximately 10 times more resistant than the parental line and shows a 10-fold cross-resistance against methotrexate, whereas vinblastine and doxorubicin are equally effective in the parental and sublines. Combined treatment of CP3 cells with cisplatin and buthionine sulfoximine (BSO) does not result in enhanced cell kill, thereby ruling out glutathione as a resistance mechanism. However, in comparison with parental cells, CP3 cells are about 1.5 times more resistant against cadmium. On the protein level, the cisplatin-resistant cells reveal an enhanced expression of metallothionein II (MTII), but not MTI, suggesting that the cisplatin resistance we observed in these sublines is at least partly mediated by MTII. These sublines will in the future serve as valuable tools for the analysis of cisplatin resistance, especially in view of metallothionein-mediated resistance mechanisms.

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Introduction

Polychemotherapy with methotrexate, vinblastine, doxorubicine and cisplatin (M-VAC) has become established in the treatment of metastatic transitional cell carcinoma (tcc). A response rate of up to 60%, with complete response of approximately 40% has been reported for this type of treatment. Unfortunately, these responses are usually of limited duration and cures are rare. No response may be expected from recurrent tumors after M-VAC therapy as they have become chemoresistant. Several different resistance mechanisms are possibly involved: vinblastine and adriamycine (doxorubicin) are substrates of the membrane-bound efflux pumps P-glycoprotein and multidrug resistance-associated protein (MRP), which remove these agents from resistant cells before they develop toxicity. Doxorubicin exerts its toxic effect by stabilizing complexes between nicked DNA and topoisomerase (topo) II, thus preventing DNA religation. Reduced activity of topo II results in doxorubicin resistance. Amplification and overexpression of the dihydrofolate reductase gene may account for methotrexate resistance.

As cisplatin is probably the most active drug in M-VAC therapy, cisplatin resistance may play a vital role in tumor recurrence. Several properties of cisplatin resistance have been described for different cell compartments. Cisplatin-resistant cell lines have been defined that accumulate less cisplatin than their sensitive counterparts. This may be attributed to the inhibition of an active cisplatin uptake mechanism [6, 18]. Membrane-bound Na/K-ATPase has been shown to be less active in resistant cells, thereby reducing the active uptake. The amount of intracellular cisplatin in sensitive cells may be reduced by 60% [17] following Na/K-ATPase inhibition by ouabain. Reduced glutathione

(GSH), its related enzymes and metallothioneins (MTs) are important cytosolic defense mechanisms that act against various toxic substances. GSH is a radical scavenger that prevents oxidative cell damage. Electrophilic substances as cisplatin are conjugated to GSH by glutathione S-transferases, which, like GSH, were overexpressed in certain cisplatin-resistant cell lines [1, 24]. MTs are small cytosolic proteins of about 6000 to 7000 kDa and are located in the cytosol and nucleus. Their physiological function is probably the regulation of intracellular zinc homeostasis and detoxification of heavy metals. The numerous thiol groups of these proteins enable the binding and detoxification of oxidizing substances. Certain authors described enhanced metallothionein expression, especially of the MTII subtype in cisplatin-resistant cells [12, 14, 19, 27]. A possible role of MT in cisplatin resistance has been demonstrated by the transfection of sensitive cells with a MTII cDNA that confers resistance to the cytotoxic action of this drug [11]. In transitional cell carcinomas, MT expression correlates with the response to cisplatin-based chemotherapy [4, 14]. Recent immunofluorescence studies demonstrated that the resistance to cisplatin correlates with nuclear MT expression and probably prevents oxidative damage to DNA [13].

Cisplatin-resistant cells also possess better tolerance towards cisplatin-DNA aducts [22] or improved reparation of cisplatin-induced DNA crosslinks [2, 7, 10, 16, 28]. No explanation exists so far for better tolerance, but several overexpressed DNA-repair genes have been found in cisplatin-resistant cells. ERCC (excision repair cross-complementing) genes belong to the latter category, particularly ERCC1.

In order to further characterize mechanisms of cisplatin resistance in urothelial cancers, we established several cisplatin-resistant sublines of the bladder carcinoma cell line RT112. We examined the cross-resistance profiles to different cytotoxic drugs and measured the expression of MTI and MTII with capillary electrophoresis.

Material and methods

Cells and cell culture

RT112 cells were generously donated by Dr. Loerke of the German Cancer Research Center, Heidelberg. The multidrug-resistant cell line RT112/D21 was established in our laboratory by culturing RT 112 cells in increasing concentrations of doxorubicin. The cisplatin-resistant cell line CPF was donated by Dr. Schuldes, Department of Urology, University of Frankfurt. All lines were kept in Dulbeccos MEM medium supplemented with 10% FCS, L-glutamine and 1% penicillin/streptomycin (10 000 IU/ml, all from GibcoBRL). Medium was changed every third day and the cells were subcultured once a week.

Chemicals and drugs

Methotrexate, vinblastine and doxorubicin were purchased from Sigma.

Establishment of cisplatin-resistant sublines

RT112 cells were first incubated in 0.5 μg/ml cisplatin (Asta Chemicals) for 4 weeks. These cells were then periodically treated with 10 μg/ml cisplatin for 2 hours followed by incubation in 0.5 μg/ml until the cells were again almost 100% confluent. This cyclic treatment was repeated five times over a period of 18 weeks. The cells were then cultured in 1.5 μg/ml cisplatin, thus generating the cisplatin-resistant subline CP1.5. On stable growth, the cisplatin concentration was increased by 0.5 μg/ml every 6 to 10 weeks. Stable sublines were acquired from each concentration increase between 1.5 and 3 μg/ml CDDP and named CP2, CP2.5 and CP3.

Cloning of cisplatin-resistant cells

CP1.5 cells were diluted in medium at a concentration of 100 cells/10 ml. Of this suspension 100 μl were pipetted into each well of a 96-well microtiter plate. The wells that contained only a single cell were marked under the microscope. The clones acquired from these wells were pipetted into a culture flask without cisplatin. Following stable growth, cisplatin was added at a concentration of 1 μg/ml and was continously increased. By this method, it was possible to establish two stable clones, K_1 and K_2.

MTT cytotoxicity assay

The yellow MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) was reduced in vital cells to a purple formazan precipitate. Cell number was counted on a hemocytometer and 2000 cells were pipetted into each well of a 96-well microtiter plate in 100 μl MEM medium. The cells were left to adhere overnight and cisplatin was then added at increasing concentrations in a volume of 100 μl medium. To achieve glutathion synthesis inhibition, BSO was added together with cisplatin at a concentration of 0.1 mM. After a 10-day incubation period, 20 μl of MTT stock solution (0.05 mg/100 ml) were added to each well. Four hours later, the liquid was removed, the formazan crystals released and then solubilized by the addition of 150 μl DMSO (Merck). The extinction of the purple color, which is directly proportional to the number of viable cells, was measured at a wavelength of 540 nm in an ELISA photometer (Titertek Multiscan Plus MKIII). The percentage of viable cells was calculated by the following formula:

$$\%\ \text{living cells} = \frac{\text{sample ext.} - \text{blank ext.}}{\text{control ext.} - \text{blank ext.}} \times 100$$

Capillary electrophoresis

After centrifugation, pellets consisting of 5 million cells were lysed in 2.1 ml of 20% trichloric acid. The resulting cell suspension was incubated with 50 μl of cadmium solution (0.2 mg/ml) and centrifuged at 400 *g* for 5 minutes. The supernatent was neutralized with 0.8 ml of 1 M TRIS-HCl pH 7.5 and 2.5 ml of this solution were loaded on PD-10 columns (Pharmacia Biotech) to remove salt and small proteins of less than 2000 Da. The resulting solution was frozen at −80°C and thawed under vacuum after 3 hours. The samples were solubilized in 400 μl demineralized water, filtered with a 30-kDa filter (Ultrafree MC, Millipore) and centrifuged for 16 minutes; 30 μl of the lower phase were used for capillary electrophoresis (PACE 2100, Beckman). The capillary had a total length of 57 cm with a detector window length of 50 cm and an internal diameter of 50 μm. The internal capillary wall was coated with hydroxy propylmethyl cellulose (Sigma) to prevent sticking of the proteins. The buffer was NaH_2PO_4-monohydrate (Merck). The samples were run for 50 minutes at 60 μA. Detection was performed with an UV-detector at a wavelength of 200 nm. The electrophoresis was calibrated with purified mouse MTII.

Results

Resistance and cross-resistance profile of cisplatin-resistant cells

Figure 1 shows killing curves of cisplatin in RT112, CP3, K1 and K2 cells. CP3 cells were the most resistant with an IC_{50} of 10 μg/ml cisplatin. The cloned sublines displayed intermediate resistance with IC_{50} values of 3 μg/ml when compared with RT112 cells with an IC_{50} of 1 μg/ml (Fig. 1, Table 1). This resistance was stable and was achieved in repeated experiments.

As M-VAC polychemotherapy is an established regimen for the treatment of bladder cancer, it was our aim to find out whether cisplatin-resistant bladder carcinoma cells display cross-resistance against the other agents administered in this type of therapy, i.e., methotrexate, doxorubicin and vinblastin. All three tested cisplatin-resistant cell lines demonstrated reproducible cross-resistance against methotrexate from 10-fold for CP3 and K2 up to 25-fold for K1 (Fig. 2, Table 1). The IC_{50} values for RT112, CP3, K1 and K2 were 2 ng/ml, 20 ng/ml, 50 ng/ml, and 20 ng/ml, respectively. Doxorubicin was slightly more effective in two of the cisplatin-resistant cells lines than it was in the parental cells (Fig. 3, Table 1). Vinblastine produced almost equal effectiveness in all tested lines (Fig. 4, Table 1).

Effect of GSH-depletion on cisplatin efficacy

The role of glutathione in the cisplatin resistance of our cell lines was investigated by performing MTT tests with increasing concentrations of cisplatin in the presence of buthionine sulfoximine (BSO, 0.1 mM), a competitive inhibitor of the rate-limiting enzyme in GSH synthesis, gamma-glutamylcysteine-synthetase. BSO co-incubation over 10 days did not alter drug resistance in the sublines CP3, K1 and K2 (Fig. 5, Table 2).

Resistance to cadmium and expression of MTs I and II

It is a well-known fact that cells expressing MTs display stronger resistance against cadmium. We measured the resistance level against cadmium in our RT112 sublines to attain an indirect estimate of MT expression. As shown in Fig. 6, CP3 cells showed a 1.5 times stronger resistance to cadmium than parental RT112 cells, thus indicating an increased expression of MTs in these cells. The relative amounts of MTI andMTII were also measured by capillary electrophoresis. Purified MTI and MTII from the mouse liver were used for reference purposes and consistently high peaks for MTI and MTII were observed after 20 and 40 minutes, respectively. After the same periods of time, small peaks were observed in RT112 cells and were arbitrarily set as 100%.

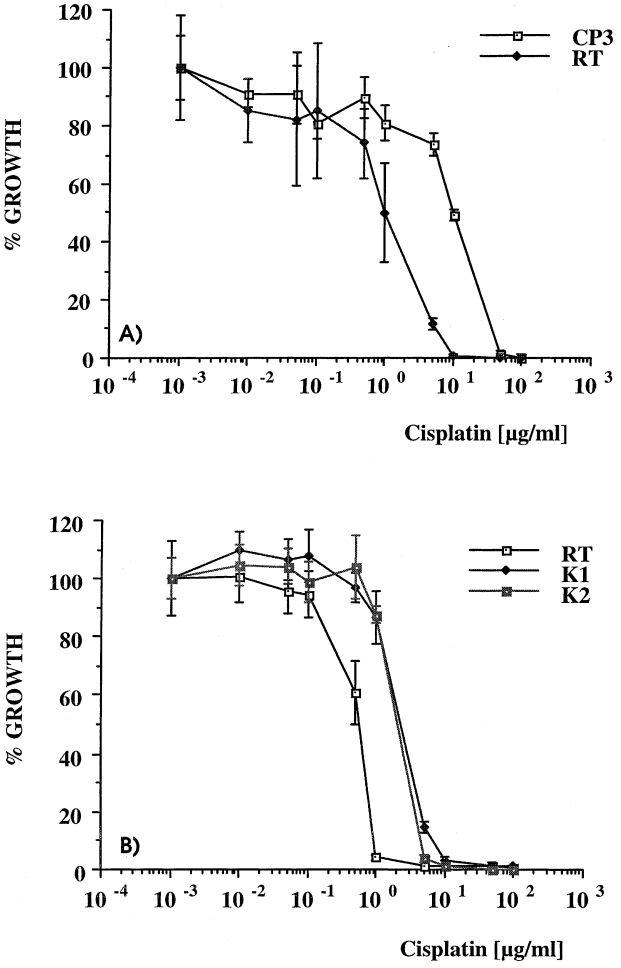

Fig. 1 Growth inhibition curves for cisplatin in the cell line RT112 and cisplatin-resistant sublines CP3, K1 and K2; 2000 cells/well, cisplatin concentrations from 0 to 100 μg/ml, incubation time 10 days

Table 1 Resistance profile of RT112 cells and cisplatin-resistant sublines K1, K2, and CP3. IC_{50} concentration of drug, resulting in a 50% growth inhibition, RF_{50} Resistance factor, IC_{50} resistant cells / IC_{50} sensitive cells

	RT112	K1	K2	CP3
Cisplatin				
IC_{50} (μg/ml)	1	3	3	10
RF_{50}	1	3	3	10
Methotrexate				
IC_{50} (ng/ml)	2	50	20	20
RF_{50}	1	25	10	10
Doxorubicin				
IC_{50} (ng/ml)	5	3	5	2
RF_{50}	1	0.6	1	0.4
Vinblastine				
IC_{50} (ng/ml)	0.003	0.004	0.004	0.005
RF_{50}	1	1.33	1.33	1.66

With increasing cisplatin resistance,the amount of MTI did not change, however, clearly rising concentrations of MTII of up to 246% (cell line CP3) were observed in our

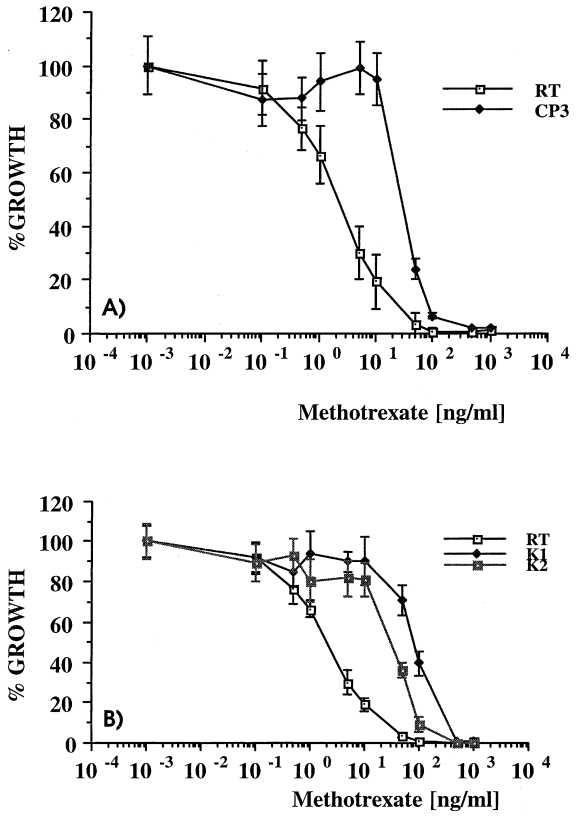

Fig. 2 Growth inhibition curves for methotrexate in the cell line RT112 and the cisplatin-resistant sublines CP3, K1, and K2; 2000 cells/well, cisplatin concentrations from 0 to 100 μg/ml, incubation time 10 days

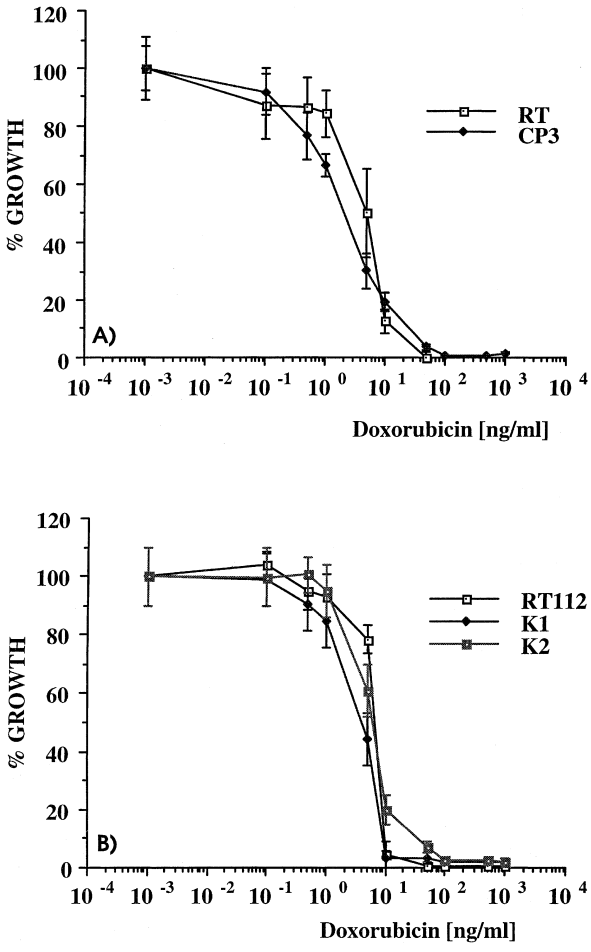

Fig. 3 Growth inhibition curves for doxorubicin in the cell line RT112 and the cisplatin-resistant sublines CP3, K1, and K2; 2000 cells/well, cisplatin concentrations from 0 to 100 μg/ml, incubation time 10 days

cisplatin-resistant sublines. On the other hand, the P-glycoprotein expressing line RT112/D21 and the cisplatin-resistant line CPF, established in continually growing cisplatin concentrations, showed only low levels of MTII (Fig. 7).

Discussion

To examine resistance mechanisms, we estabished cisplatin-resistant sublines of the bladder carcinoma cell line RT 112. The most resistant subline tolerated a 10-fold higher cisplatin concentration, than the parental line, which is low compared with other selected cisplatin-resistant lines [23]. With our system, we attempted a simulation of the clinical situation with low-level drug resistance. Furthermore, cisplatin is a very potent mitogen that might induce a high mutation rate which may increase with drug concentration in the medium and lead to numerous genetic changes in highly resistant cells that have nothing to do with cisplatin resistance [3]. The detection of clinically important and early defence mechanisms can therefore be hampered in these cells.

With substances from the M-VAC polychemotherapy regimen, we investigated the resistance profile of our cells and found a consistent cross-resistance to methotrexate. This cross-resistance pattern has been described for other cisplatin-resistant cell lines and reports have been made of early occurrence in cells that display only low cisplatin resistance. Over 30 years ago, the main mechanism of methotrexate resistance was described as the fast amplification and overexpression of the diyhydrofolate reductase gene (DHFR gene). This mechanism plays no obviously important role in cisplatin-resistant cells showing cross-resistance to methotrexate. It was recently suggested that an altered methotrexate uptake and not overexpression of the DHFR gene can be found in cells selected for cisplatin resistance [25]. Whatever the mechanism of methotrexate cross-resistance might be, it is obviously of great clinical relevance, since both drugs belong to the M-VAC regimen. Cisplatin-resistant bladder carcinomas can also become resistant to methotrexate, thus inhibiting the curative effect of this chemotherapy.

Doxorubicin and vinblastin also share one common drug resistance mechanism as they are substrates of the drug transporter P-glycoprotein, frequently overexpressed in vinblastin- and doxorubicin-resistant cells.

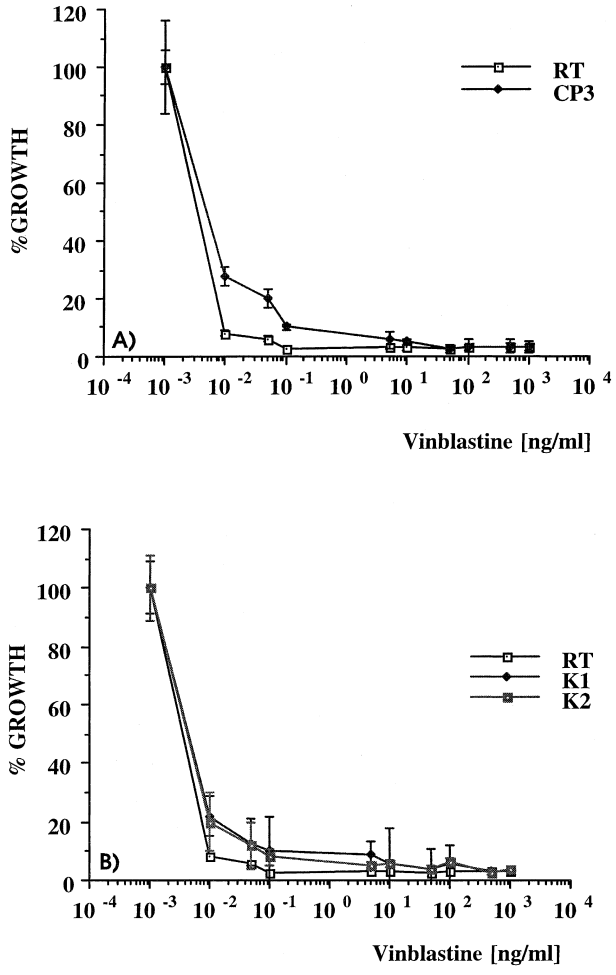

Fig. 4 Growth inhibition curves for vinblastine in the cell line RT112 and the cisplatin-resistant sublines CP3, K1, and K2; 2000 cells/well, cisplatin concentrations from 0 to 100 µg/ml, incubation time 10 days

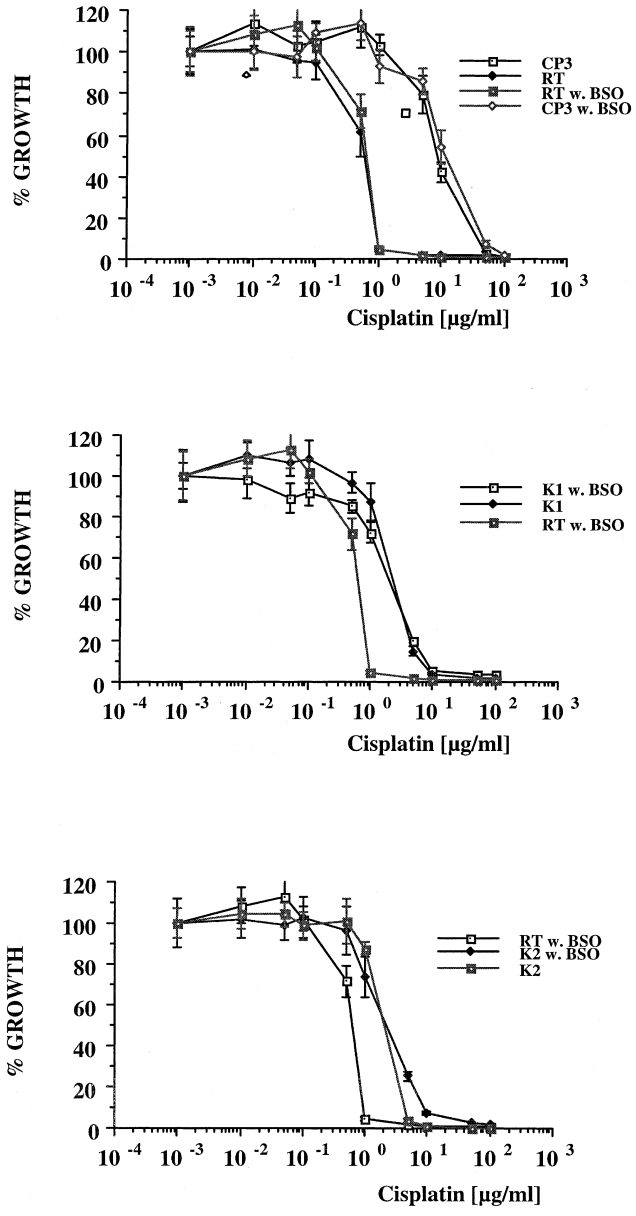

Fig. 5 Growth inhibition curves for cisplatin with and without BSO in the cell line RT112 and the cisplatin-resistant sublines CP3, K1, and K2; 2000 cells/well, cisplatin concentrations from 0 to 100 µg/ml, incubation time 10 days

Immunohistochemistry easily demonstrates the presence of P-glycoprotein in a doxorubicin-resistant subline of RT112, RT112/D21 and absence in cisplatin resistant sublines. Tumor cells can therefore express different resistance mechanisms, depending on exposure to the various cytotoxic substances. By the expression of two different resistance pathways, bladder carcinomas could become resistant to all four drugs used in M-VAC therapy.

Enhanced concentration of glutathione has been repeatedly described in different cisplatin-resistant cell lines, e.g., by Bedford et al. [5] in a cisplatin-resistant RT112 subline. Compared with the parental line, they found a 1.5-fold enhanced concentration of GSH in the resistant subline – an important resistance factor in their opinion. BSO shows structural similarity to glutamate, one of the substrates of gamma-glutamylcystein-synthetase, the key enzyme in GSH synthesis. This similarity makes it a competitive enzyme inhibitor, resulting in depletion of the intracellular GSH-pool. The activity of BSO is schedule dependent. In gastric carcinoma cell lines, Saikawa et al. [21] demonstrated that prior to

Table 2 Effect of BSO on cisplatin resistance of RT112 cells and cisplatin-resistant sublines K1, K2 and CP3

	RT112	K1	K2	CP3
IC_{50}/BSO (µg/ml)	0.7	2.2	2.0	10
IC_{50} (µg/ml)	0.7	2.2	2.0	12

cisplatin treatment a 24-hour pre-incubation with BSO enhanced cisplatin efficacy more potently than simultaneous treatment or BSO incubation subsequent to cisplatin. However, all treatment schedules reduced the viablility of the treated cells to less than 30% of the

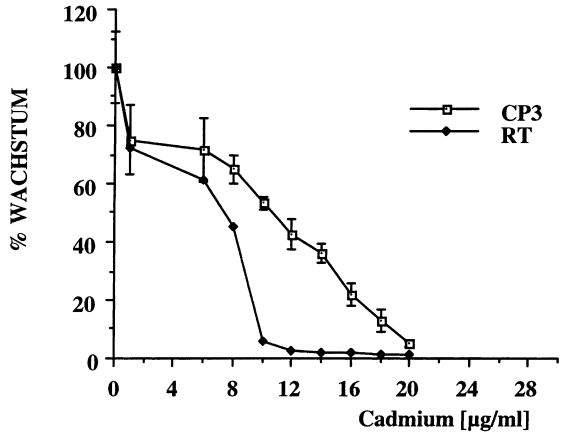

Fig. 6 Growth inhibition curves for cadmium in the cell line RT112 and the cisplatin-resistant subline CP3; 5000 cells/well, cadmium concentrations ranged from 0 to 20 µg/ml, incubation time 6 days

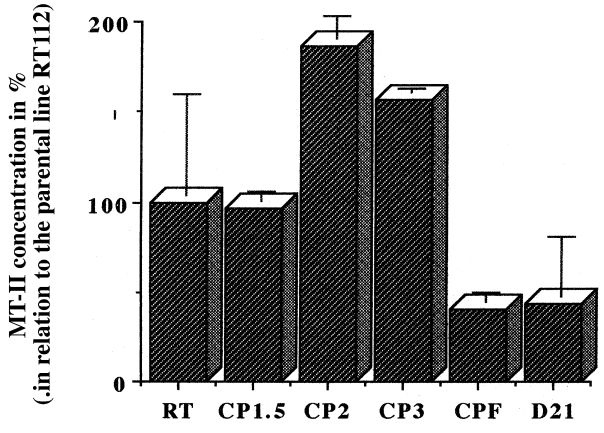

Fig. 7 Relative concentration of MTII in RT112 cells and different cisplatin-resistant sublines. *CP1.5*, *CP2*, *CP3*, *CPF* cisplatin-resistant RT112 sublines, *RT*, *D21* doxorubicin-resistant subline

untreated resistant cells. To exclude an enhanced GSH concentration as a possible resistance mechanism we coincubated our cisplatin-resistant cells for 10 days in cisplatin and BSO. Over a relatively long incubation period, no difference in the resistance level with or without BSO was observed, and in our opinion enhanced GSH production as a resistance factor in our cells was ruled out.

It has been demonstrated that cisplatin can induce MTs and several reports were made on enhanced MT levels in cisplatin-resistant cell lines [13, 14, 20]. MTs have been divided into the two classes MTI and MTII. They possess high polymorphism, especially in the human genome, where 10 isoforms have been detected [9]. Cisplatin resistance seems to result from overexpression of MTII as no reports exist on the connection with MTI overexpression. CP3 cells showed an enhanced resistance against cadmium suggesting an increased expression of MTs. We confirmed this by capillary electrophoresis and further demonstrated that progressively increasing resistance to cisplatin in our RT112 sublines correlates with an over two-fold MTII expression in comparison with the parental line. Together with evidence presented in the literature that MTII is a resistance factor against cisplatin, our results suggest that this MT isotype also plays an important part in the cisplatin resistance of our cell lines [11].

There is no conclusive reason why some resistant sublines, even from the same parental line, inactivate cisplatin with MTII, while others express higher levels of GSH. A possible explanation could be the rapid induction of MTs by cisplatin. A 1.6-fold induction of MTII can be assessed as soon as 2 hours after cisplatin treatment of RT112 cells (unpublished results). It is therefore possible that a protocol for selection of cisplatin-resistant cells with multiple short-term cisplatin incubations with high drug concentrations might favor MT induction, whereas selection in slowly increasing cisplatin concentrations might result in higher concentrations of GSH. This hypothesis is supported by the results of Bedford et al. [5] who described enhanced GSH concentration in cisplatin-resistant RT112 cells after exposure to slowly increasing drug concentrations. The RT112 cisplatin-resistant subline CPF was also established in steadily increasing cisplatin concentrations and probably as a result of this protocol expresses only a low amount of MTII, but overexpresses GSH.

In conclusion, we established cisplatin-resistant sublines of the bladder carcinoma cell line RT112 with low to moderate resistance levels. These sublines show cross-resistance to methotrexate and this implies that the cross-resistance profile develops at an early stage of induction of cisplatin resistance. This could be of great clinical relevance, since both drugs are components of the M-VAC therapy. Further characterization of our cisplatin-resistant sublines revealed an overexpression of MTII, a likely important resistance mechanism. These cells will be of great value in further investigations attempting to uncover new mechanisms of cisplatin resistance.

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