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Establishment and characterization of a multidrug-resistant human bladder carcinoma cell line RT112/D21

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Abstract A doxorubicin-resistant human bladder carcinoma cell line RT112/D21 was established by continuous exposure of the parental line RT112 to increasing concentrations of doxorubicin over a period of 9 months. RT112/D21 cells expressed significantly more P-170 glycoprotein than the parental line, and rhodamine 123 efflux, as a functional parameter of P-170 glycoprotein activity, was increased. RT112/D21 cells were 96 times more resistant to doxorubicin than RT112 cells, and cross-resistance to epirubicin and vinblastine was present. Sensitivity to methotrexate and mitomycin C remained unchanged. R-verapamil reversed resistance to doxorubicin, epirubicin and vinblastine in RT112/D21 cells but did not affect sensitivity to methotrexate and mitomycin C. In RT112 cells, R-verapamil had no effect on drug sensitivity. Thus, it may be assumed that primary or induced *MDR1* gene-encoded P-170 glycoprotein expression is a relevant mechanism of chemoresistance in transitional cell carcinoma, and that chemotherapeutic strategies in combination with chemosensitizers improve response rates.

Key words Bladder cancer · Doxorubicin
Multidrug resistance · P-170 glycoprotein
Rhodamine 123 · R-verapamil

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In the management of transitional cell carcinoma of the bladder, chemotherapy has been widely accepted as an adjuvant treatment to surgical resection. The treatment of choice in superficial malignancies is endoscopic resection followed by intravesical chemo- or immunotherapy for high-risk tumors. However, tumor recurrence is predicted in up to 30% of all patients and progression is reported in 7% of cases [16, 36], indicating resistance to the chemotherapeutic agent in a substantial number of tumors.

Treatment of advanced (N+) or metastatic (M+) transitional cell carcinoma with polychemotherapy as M-VAC or M-VEC achieved complete remission in 32% and 23% of cases, respectively [41]. An initial response followed by a relapse in 60–70% of cases is a common phenomenon during treatment. This indicates that chemosensitive tumors acquire drug resistance during chemotherapy, either by selection of primarily resistant subclones or by induction of a resistance mechanism in primarily sensitive cells. A well-characterized mechanism of drug resistance is the expression of the *MDR1* gene-encoded P-170 glycoprotein (P-GP). P-GP functions as an energy-dependent efflux pump providing chemoresistance to a variety of structurally unrelated cytotoxic drugs such as the vinca alkaloids or anthracyclines by active transmembranous transport [11, 19, 33].

P-GP expression in normal bladder mucosa is low [2]. However, a previous study has shown that untreated transitional cell carcinoma of the bladder expressed P-GP in 31.6% of cases, and chemosensitivity to Adriamycin and vinblastine in an ex vivo oncobiogram was significantly correlated with P-GP expression [2]. Thus, it may be speculated that acquired chemoresistance of urothelial tumors is related to overexpression of P-GP [2, 31]. We therefore developed a doxorubicin-resistant human bladder cancer cell line (RT112/D21) and compared the resistant subline with the sensitive parental line in terms of P-GP expression, P-GP function and cross-resistance to different chemotherapeutic agents. Additionally, the effect of the chemosensitizer R-verapamil was assessed.

Materials and methods

Cell culture and induction of drug resistance

The urothelial carcinoma cell line (RT112) was kindly provided by the German Cancer Research Institute, Heidelberg. Cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2% glutamine and penicillin/streptomycin (all purchased from Gibco). A parental stock sample was frozen in 10% dimethyl sulfoxide (DMSO) and kept at −80 °C. The resistant subline (RT112/D21) was established by exposure of the cell culture to doxorubicin over a continuous period of 9 months. The initial doxorubicin concentration was 0.01 μg/ml medium, drug concentration was increased gradually in 0.005-μg/ml steps when passage periods were 1 week or less. After reaching a final concentration of 0.11 mg doxorubicin/ml medium, the resistant subline was compared with the parental stock sample.

Immunohistochemical assessment of P-GP expression

Cells were cultured on glass slides. On 50% confluency, they were fixed in cold acetone (10 min), air dried and stored until analysis at −20 °C. The P-GP expression was detected immunohistochemically using the specific anti-P-GP antibody mAb C219 (Centocor) and the biotin streptavidin technique. An immunoreactive score (IRS) was used for quantification of the P-GP immunoreactivity. This score was defined in accordance with that of Remmele et al. [37] for quantification of estrogen receptors in breast carcinoma. The average quantity of P-GP-positive carcinoma cells per microscopic high-power field per slide was multiplied by the staining intensity (+ = 1; ++ = 2; +++ = 3), resulting in a semiquantitative IRS ranging from 0 to 12.

Functional assay of P-GP with rhodamine 123 (R123)

The rhodamine assay is based on the transportation of the fluorescent dye R123 by P-GP [7, 23]. For the R123 efflux experiments, five samples with 10^6 cells each were preincubated for 15 min at 37 °C with or without R-verapamil 5 μg/ml (Knoll), 1 μg/ml R123 (Sigma) was added and the samples were incubated for another 15 min in the dark. Subsequently the cells were washed twice with RPMI and a sample was kept at 4 °C on RPMI until FACS (Fluorescence activated cell measurement system) analysis for the assessment of R123 influx. The other samples were incubated in R123 free medium for 5, 15, 30 and 60 min (R123 efflux analysis). The samples were then washed with ice-cold RPMI, resuspended and kept at 4 °C until FACS analysis, which was performed on a Becton Dickinson flow cytometer. R123 fluorescence was analyzed at 530 nm. R123 enters vital cells and stains mitochondria with moderate affinity. Efflux, due to loss of cell membrane integrity of dead cells, was excluded by counterstaining with propidium iodine (fluorescence at 580 nm), which stains dead cells only.

Drug resistance assay

Drug resistance screening was performed using the microculture tetrazolium assay [1, 27, 30]. This test is based on the conversion of the yellow tetrazolium salt to a blue formazan precipitate by viable cells. The formazan crystals are solubilized and extinction is read at 540 nm. Briefly, for analysis of cell viability, 20 μg/100 μl MTT (Sigma) was added to the test plates for 4 h to allow conversion to formazan. The plates were centrifuged at 1200 rpm for 5 min and the supernatant was decanted. After solubilization of the formazan precipitate with 100 μl DMSO, extinction was read within 15 min on a microculture plate reader (Titertec Multiscan Plus MKII). For the evaluation of cell response to drugs, 96 flat-bottomed well microtiter plates were inoculated with 5000 cells/well and incubated for 24 h prior to drug addition. After this preincubation period, one plate of each cell line was analyzed for viable cells as time zero value (T_z). Subsequently, the test substances doxorubicin, epirubicin, vinblastine, methotrexate and mitomycin C were added in increasing concentrations with or without 5 μg/ml R-verapamil and the cells were incubated for another 6 days until final analysis of cell growth (T_6).

Drugs

Doxorubicin (Adriamycin, Farmitalia), epirubicin (Farmarubicin, Farmitalia), vinblastine (Velbe, Lilly), methotrexate (Lederle) and mitomycin C (Medac) were prepared in accordance with the manufacturer's instructions and serially diluted in medium just before addition to the test plates. R-verapamil (Dexverapamil, Knoll) was freshly diluted prior to each experiment. Samples were run in triplicate, values were calculated in accordance with the formula $((T_6-T_z)/T_z)$ 100, indicating the percentage of the overall cell growth and cell kill. The drug concentration effecting a 50% inhibition of cell growth was regarded as the IC_{50}. Relative resistance of RT112/D21 cells to a test substance was calculated by IC_{50} RT112/D21/IC_{50} RT112.

Results

Growth characteristics

The cell growth of the parental line RT112 was slightly faster than the growth of the subline RT112/D21. Doubling times were 27 and 29 h for RT112 and RT112/D21, respectively. Cell morphology of RT112/D21 was more inhomogeneous than of RT112, and RT112/D21 cells appeared larger.

Immunohistochemical analysis of P-GP expression

The immunostaining pattern of P-GP was membranous or cytoplasmatic in both parental RT112 and RT112/D21 cells. However, in most RT112 cells, P-GP was absent and only 5% of the cells showed a low staining intensity. The IRS of RT112 cells was 2. In contrast, almost all RT112/D21 cells stained positive, and 30% with particularly intense staining, resulting in an IRS of >8.

Functional rhodamine 123 assay (Fig. 1)

R123 uptake in parental RT112 cells was significantly higher than in the subline RT112/D21. R-verapamil increased dye accumulation of resistant RT112/D21 cells to the level of parental RT112 cells but failed to alter R123 uptake in RT112 cells. RT112 cells also retained R123 during the 60-min control period while RT112/D21 cells rapidly decreased intracellular R123 concentration after 5 min. In the presence of R-verapamil, the dye concentration remained unchanged in RT112 cells during the 60-min control period. In resistant RT112/D21 cells, R123

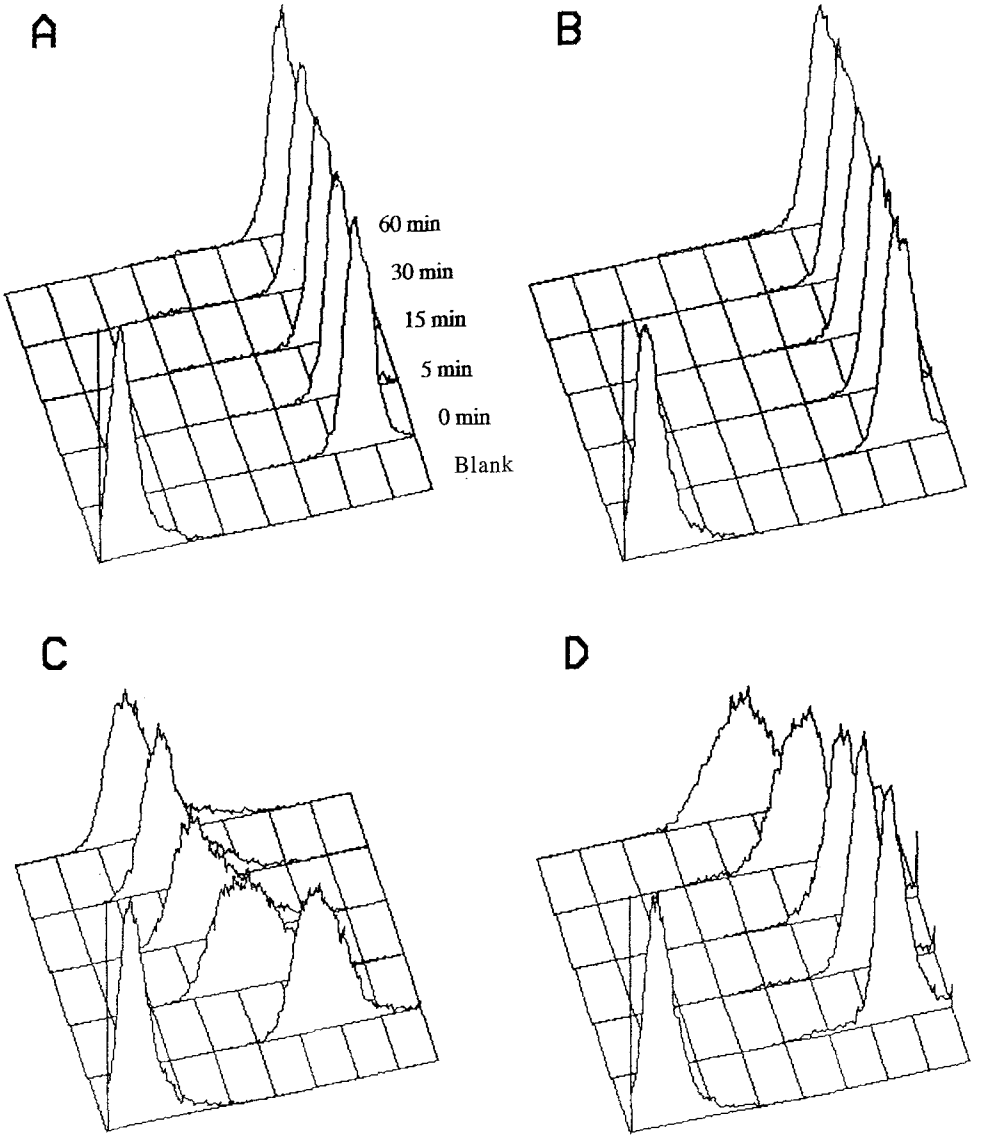

Fig. 1A–D Fluorescence histograms showing time-dependent (0, 5, 15, 30 and 60 min) rhodamine 123 dye exclusion of RT112 and RT112/D21 cells ± R-verapamil: **A** RT112 without R-verapamil. **B** RT112 cells with R-verapamil. **C** RT112/D21 cells without R-verapamil. **D** RT112/D21 cells with R-verapamil

efflux was inhibited up to 15 min while intracellular dye reduction was slightly visible at 30 min and more pronounced at 60 min.

Drug resistance

The resistance profile of the parental and subline was screened against doxorubicin, vinblastine, methotrexate and mitomycin C alone or in combination with R-verapamil. Growth inhibition curves of the test substances are shown in Fig. 2. RT112/D21 cells exhibited increased resistance to doxorubicin, epirubicin and vinblastine whereas the sensitivity to methotrexate, mitomycin C and R-verapamil was unchanged and comparable to that of the parental line. R-verapamil alone led to growth inhibition at concentrations over 50 μg/ml. In the presence of 5 μg/ml R-verapamil, the increased drug resistance of RT112/D21 cells was reversed by more than 96%. In RT112 cells, incubation with 5 μg/ml R-verapamil increased sensitivity to doxorubicin, epirubicin and vinblastine by the factors 5.0, 4.5 and 1.2. Drug toxicity of methotrexate and mitomycin C remained unaltered in the parental line and subline when incubated with 5 μg/ml R-verapamil. The IC_{50} values and relative resistance to the tested drugs with and without addition of R-verapamil are shown in Table 1.

Table 1 IC_{50} values (mg/ml) of RT112 and RT112/D21 ± R-verapamil (R-VPM) for the different test drugs; the resistance factor expresses the degree of chemoresistance of RT112/D21 cells compared with RT112 cells

Substance	RT112	RT112 +R-VPM	RT112/D21	RT112/D21 +R-VPM	Resistance factor
Doxorubicin	0.0045	0.00094	0.43	0.015	96
Epirubicin	0.0037	0.0008	0.49	0.0071	132
Vinblastine	0.0004	0.00034	0.067	0.00034	168
Methotrexate	0.0135	0.0135	0.0125	0.0125	0.9
Mitomycin C	0.035	0.035	0.035	0.035	1

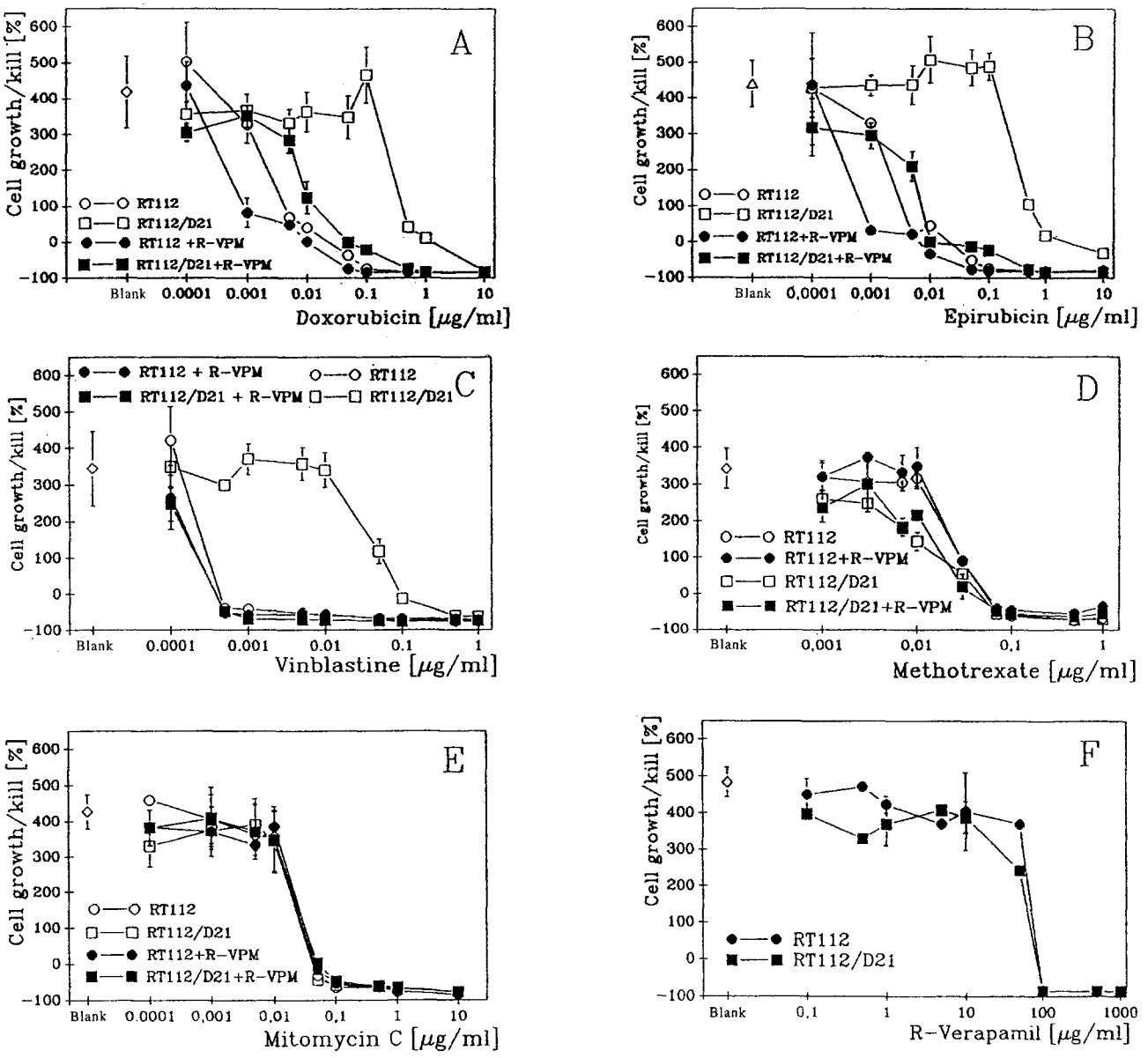

Fig. 2A–F Growth inhibition curves of RT112 and RT112/D21 for doxorubicin (**A**), epirubicin (**B**), vinblastine (**C**), methotrexate (**D**), mitomycin C (**E**) and R-verapamil (**F**). Growth inhibition in graphs **A–E** was tested in the absence (*open symbols*) and presence (*filled symbols*) of 5 μg/ml R-verapamil. *x-Axis* test drug concentration (logarithmic scale), *y-Axis* cell growth/kill after 6 days incubation as percentage of day 1 value

Discussion

The phenotype of pleiotropic drug resistance or multidrug resistance (MDR) has been extensively described as a common phenomenon of malignant tumors in vivo and in vitro mediated by the expression of *MDR1* gene-encoded P-GP, which serves as a transmembrane pump, reducing the intracellular accumulation of drugs [22, 26, 29].

In the current study, a bladder cancer cell line was continuously exposed to doxorubicin in order to induce chemoresistance. Cells of the resistant subline RT112/D21 exhibited significantly more P-GP than sensitive RT112 cells. Effert et al. [7] demonstrated that the degree of P-GP expression correlates with drug efflux [7] and several markers have been described for the determination of intracellular drug accumulation/drug efflux as a functional assay of P-GP [7, 17, 23]. We used the fluorescent dye R123 in a flow cytometric assay in accordance with the procedure previously described by Ludescher [23]. In accordance with the P-GP staining, RT112/D21 cells exhibited reduced R123 uptake and increased efflux compared with parental RT112 cells.

Drug resistance to a panel of different chemotherapeutic agents was tested in a colorimetric assay. Compared with the parental line, the resistant subline RT112/D21 was 96 times more resistant to doxorubicin. This corresponds with the findings of other investigators, who

achieved 9- to 271-fold doxorubicin resistance in urothelial cancer cell lines [9, 20, 25]. RT112/D21 cells were cross-resistant to epirubicin as well as to the chemically different vinca alkaloid vinblastine, but remained sensitive to methotrexate and mitomycin C. It has been suggested that the resistance to mitomycin C is mediated by the classical MDR phenotype [26]. Our results did not confirm this but Kimiya et al. described similar unaltered sensitivity to mitomycin C of a multidrug-resistant cell line [20]. Thus, it may be speculated that resistance to mitomycin C is mainly based on mechanisms other than P-GP.

Based on the drug resistance pattern, the positive immunohistochemical P-GP reaction and the altered drug accumulation/efflux, we considered the subline RT112/D21 as a cell line with classical MDR which was subsequently tested for MDR reversal.

There are several known methods of reversing P-GP mediated MDR. However, most of them are still experimental and unsuitable for clinical use. Anti-sense oligodeoxynucleotides preventing P-GP expression at the mRNA transcription level [38] and conjugation of a toxin to a monoclonal antibody against P-GP have been shown to specifically kill MDR cells [8]. Much effort has been focussed on the pharmacological antagonization of MDR. In 1984 Simpson et al. [40] demonstrated an increased cytotoxic effect of doxorubicin on human bladder cancer cells when administered together with verapamil, although the precise mechanism of this phenomenon had not been elucidated at that time. Today, the calcium antagonist verapamil is one of the best investigated substances for the reversal of MDR [10]. We used R-verapamil in our experiments as this isomer is less cardiodepressive in a systemic application [6]. R-verapamil was toxic to the cell culture at concentrations of more than 50 μg/ml. At a concentration of 5 μg/ml, drug sensitivity to MDR substances was slightly increased in parental RT112 cells. This is explained by the immunohistochemically shown P-GP expression in 5% of these cells. In resistant RT112/D21 cells, the 168-fold chemoresistance to vinblastine was completely reversed by incubation in the presence of 5 μg/ml R-verapamil. Resistance to epirubicin and doxorubicin was decreased from 132-fold to 1.9-fold and from 96-fold to 3.3-fold, respectively. This, however, indicates that RT112/D21 cells also acquired a resistance mechanism different to P-GP.

In fact, several other mechanisms of drug resistance are known. The glutathione redox cycle is involved in detoxification of many cytotoxic agents including chemotherapeutics [26, 42]. DNA topoisomerases are the target of doxorubicin, etoposide and mitoxantrone, and low levels of expression are associated with resistance [15]. An additional resistance mechanism was postulated when drug-resistant cell lines were described that exhibited a cross-resistance profile comparable to the classical MDR type but with no overexpression of the *MDR1* gene [32, 4]. Indeed, a multidrug resistance-associated protein (MRP) was isolated which has only the predicted ATP-binding domain in common with P-GP; however, chemoresistance mediated by MRP was very similar to classical MDR [5].

In the present study, the subline was not analyzed for resistance mechanisms different to classical MDR. Thus, a participation of one or more of these mechanisms in the resistance profile of RT112/D21 cells cannot positively be excluded. However, several facts suggest that drug resistance of the RT112/D21 subline is mainly based on the *MDR1* gene-encoded classical MDR phenotype: In tumor cells with both elevated P-GP and glutathione expression, it has been reported that resistance to doxorubicin was completely reversed when blockers of both mechanisms, i.e., verapamil and buthionine sulfoximine (BSO), were combined [27, 35]. Modulation of doxorubicin resistance was incomplete with the sole administration of verapamil, indicating that verapamil has no effect on glutathione-mediated resistance. In our RT112/D21 subline, the administration of R-verapamil reversed 100% and 96% of the resistance to vinblastine and doxorubicin, respectively. This suggests that no more than 4% of the doxorubicin resistance is mediated by increased glutathione metabolism. Moreover, it has been suggested that glutathione mediates resistance to platinum compounds [27]. In RT112/D21 cells, sensitivity to cisplatin was not changed in comparison to the parental line (results not shown), indicating that glutathione expression is not significantly increased in our resistant subline.

Resistance to doxorubicin is also described in cells with reduced expression of topoisomerase II. However, topoisomerase as a target of the chemotherapeutic agent is very unlikely to mediate a reduction of the intracellular drug concentration as shown in the RT112/D21 cell by means of the rhodamine 123 assay. In addition, Hill et al. reported on two P-GP-negative, but drug-resistant tumor sublines characterized by altered topoisomerase II activity, which did not show resistance modulation upon verapamil administration [18]. Obviously, verapamil does not have a significant impact on DNA topoisomerase. Thus, it is concluded that this particular resistance mechanism does not play a major role in RT112/D21 cells since drug resistance in this subline was almost completely circumvented by incubation with R-verapamil.

Another non-P-GP resistance mechanism is MRP as previously described by Cole et al. [5]. In MRP-positive cells, reduced drug accumulation appears to be related to increased drug efflux or to a mechanism for sequestering drugs away from the cytotoxic target. However, it is controversial whether MRP is modulated by verapamil. Grant et al. [12] isolated and cloned sequenced cDNA from drug-resistant H69AR cells which proved to encode MRP. This particular line was described earlier to display a resistance profile similar to classical MDR without overexpression of *MDR1*, and MDR was poorly reversed by chemosensitizers that are effective in cells overexpressing P-GP [28, 5]. In contrast, Kruh et al. [21] found that verapamil can reverse the drug-resistant phenotype of MRP transfectants, analogous to the effect of this drug on cells that overexpress P-GP. In our resistant cell line, drug resistance was almost completely reversed by vera-

pamil. Since MRP, similar to P-GP, may be a transporter protein, the rhodamine 123 exclusion assay is not suitable to pinpoint P-GP as the basic resistance mechanism in RT112/D21 cells. Grogan et al. [13] described a linear correlation between P-GP expression and drug resistance with an array of different resistance levels of a myeloma cell line which was gradually selected for resistance to doxorubicin [13]. In the present study, immunohistochemistry was not evaluated by a computerized cell analysis system but subjectively rated and only one resistance level, namely that of RT112/D21 cells, was analyzed. A standard curve for correlation of P-GP expression with drug resistance could therefore not be obtained. However, we found a significant increase in P-GP staining intensity, which rose from 2 to 8 in a range of 12 arbitrary units. In the parental line, 95% of the cells stained negative while all evaluated cells in the subline stained positive. This indicates that classical MDR was present in all RT112/D21 cells. However, participation of MRP in drug resistance of the subline cannot be completely ruled out. Cloning of RT112/D21 cells and MRP analysis will be the subject of further investigation to clarify this question.

In the management of superficial bladder carcinoma, chemoprophylaxis with mitomycin C proved to be very effective [36]. However, recurrence is reported in 26% of patients, indicating the necessity for a powerful second-line strategy. In our cell line, mitomycin C relied on a different resistance mechanism than anthracyclines. Treatment with doxorubicin or epirubicin could therefore be effective in patients with resistance to mitomycin C. This is supported by the results of Sekine et al., who achieved an initial complete response rate of 74% in patients with grade 3 carinoma in situ of the bladder using sequential instillation therapy with mitomycin C/doxorubicin [39]. However, 41% of the initial responders relapsed in further follow-up. On the other hand, it has been suggested that bladder tumors may also express P-GP [2, 31] mediating resistance to anthracyclines. In our in vitro experiment, chemoresistance of the bladder cancer cell line RT112/D21 to anthracyclines was reversed by R-verapamil. Several investigators have described the intravesical administration of Adriamycin or epirubicin in combination with racemic verapamil for prophylaxis of Ta/T1 tumors. Ohi et al. [34] did not achieve an increased antitumor effect of Adriamycin plus verapamil, while Lukkarinen et al. [24] reported a slight, but not significant, reduction of tumor recurrence when epirubicin was combined with verapamil. The latter investigators also demonstrated that verapamil increased epirubicin concentration in the tumor tissue. However, only 30% of urothelial bladder carcinomas primarily express P-GP [29, 31], but tumors were not analyzed for P-GP prior to treatment. Thus, it is assumed that some patients treated with verapamil had P-GP-negative tumors. Therefore, the assessment of P-GP expression in tumors prior to treatment is advisable as only P-GP-positive tumors respond to the chemosensitizer.

Benson et al. [2] previously demonstrated that P-GP expression seems to be higher in deeply invasive bladder tumors than in superficial tumors. In a limited series, Naito [31] et al. found that all patients pretreated with M-VAC for transitional cell cancer expressed P-GP and tumor cells were resistant to doxorubicin and vinblastine in an in vitro chemosensitivity test. A concentration of 2–6 μM is required for systemic antagonization of MDR with racemic verapamil [43]. This is 2–6 times higher than tolerated with respect to serious cardiovascular side effects. R-verapamil is equieffective as resistance modulator [14] but ten times less cardiodepressive than racemic verapamil. In a clinical phase I pharmacokinetic study it was shown that plasma concentrations up to $\sim 4\ \mu M$ (~ 2 μg/ml) can be achieved under adequate patient monitoring [3]. Therefore, patients with invasive bladder carcinoma might respond to systemic chemotherapy in combination with R-verapamil even after relapse to polychemotherapy containing MDR substances such as doxorubicin and vinblastine.

The cell line presented in this study exhibited a similar drug resistance profile to that reported previously by others [9, 20, 25]. This suggests that, in the model of bladder cancer cell cultures, development of drug resistance occurs in a reproducible rather than an accidental manner. This is especially important when in vitro results are to be clinically applied. However, more studies will be needed to further elucidate the relevance of P-GP in transitional cell carcinoma. The MDR cell line RT112/D21 is considered a valuable standard for further in vitro experiments in this field as well as for testing and development of drugs in the search for new chemosensitizers.

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