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Induction of drug-resistant bladder carcinoma cells in vitro: impact on polychemotherapy with cisplatin, methotrexate and vinblastine (CMV)

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Abstract Residual tumor, tumor progression or relapse after chemotherapy of patients with advanced or metastasized transitional cell carcinoma of the bladder (TCCB) are suggested to reflect intrinsic drug resistance of cancer cells, or the development of chemotherapy-resistant tumor cell populations. The present study aimed to establish drug-resistant subculture cell lines from human TCCB, selected for anticancer drugs, administered in the cisplatin, methotrexate and vinblastine (CMV) polychemotherapy protocol. Tumor cells from chemonaive cell lines of human TCCB (HT1376, TCCSUP) have been exposed to progressively increasing concentrations of cis-diamminedichloroplatinum (II) (CDDP), methotrexate (MTX), vinblastine (VBL) or etoposide (VP16). The resulting drug-resistant subculture cell lines (HT1376-CDDP, HT1376-MTX, HT1376-VBL, HT1376-VP, TCCSUP-CDDP, TCCSUP-MTX, TCCSUP-VBL, TCCSUP-VP) were analyzed with regard to the achieved resistance factor (RF) for the inductive anticancer agent, the acquisition of cross-resistance, DNA content, cell cycle distribution and cellular morphology. Parental HT1376 cells were intrinsically less sensitive to all anticancer drugs (1.7–50×), compared with TCCSUP cells. Relative resistance against the inductive anticancer agents was similar for the final drug-resistant subculture cell lines of both parental cell lines concerning CDDP and VP-16 (RF: 4–5×), but were reciprocal for MTX and VBL, respectively. MTX led to much stronger resistance (RF > 200) than the other drugs (RF < 10). Pleiotropic cross-resistances were observed in six out of eight (75%) drug-resistant subculture cell lines. Highest RF (50–500×) and frequency of cross-resistance (five of six cell lines) occured for MTX, and the least from exposure to CDDP (one of six cell lines). Overall, the results corroborated the central role of CDDP against urothelial carcinoma whereas repetitive applications of MTX appeared to be a doubtful strategy. Moreover, the experiments provide the largest panel so far of drug-resistant cell lines of human TCCB. They represent an appropriate tool for basic research on drug-resistance mechanisms, for the development and screening of future anticancer drugs or to elaborate strategies to overcome drug resistance for those patients who ultimately fail to respond to standard chemotherapy.

Key words Urothelial carcinoma · Drug resistance · CMV · Chemotherapy · Cell culture

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Introduction

Urothelial carcinomas are sensitive to chemotherapeutic agents with different structural and functional properties. Cisplatin (CDDP) was suggested to be the most active single anticancer agent for patients with advanced or metastatic transitional cell carcinoma of the bladder (TCCB) effecting remission rates of 17%–34% [34]. Monochemotherapy with methotrexate (MTX) led to comparable overall response rates [49]. In contrast, monochemotherapy with vinblastine (VBL) resulted in minor responses (16%) [48]. Other agents with single drug activity against transitional cell carcinoma include adriamycin (doxorubicin), 5-fluorouracil, carboplatin, mitomycin-C, cytoxan or etoposide. Polychemotherapy appeares to increase the response rates and prolongs survival compared with monochemotherapy [49]. Among several different regimens, combinations of cisplatin with methotrexate (CM), vinblastine (CMV) [15] or adriamycin (MVAC) [41], are actually the most common. Those combinations led to complete response rates up to 30% and overall response rates in excess of 70% [5, 14, 17, 41, 43]. However, relapse after initial response to chemotherapy occurs in approximately

60%–70% [35]. Several attempts have been made to influence tumor progression by different second-line therapies [18, 44]. One particular reason for tumor progression or tumor relapse is likely to be an intrinsic or acquired resistance of tumor cell populations to anticancer drugs.

Only a few experiments have been performed to induce drug-resistant tumor cell lines from TCCB in the past. The present study aimed to establish new drug-resistant cell lines from chemonaive human TCCB. The in vitro experiments focused especially on the activity of the single anticancer agents, that are administered in the CMV polychemotherapy protocol (CDDP, MTX, VBL), with regard to their capacity to induce resistance and cross-resistance. In addition, the activity of etoposide (VP16) was investigated.

Material and methods

Tumor cell culture

Parental cell lines were derived from human chemonaive transitional cell carcinoma. HT1376 [31] and TCCSUP [25] cells were maintained in cell culture flasks under identical, sterile cell culture conditions (7% CO_2, 37°C). Standard cell culture medium, Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Paisley, UK), was supplemented with 15% (v/v) heat-inactivated fetal calf serum (FCS; Gibco BRL), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Gibco BRL). Cell cultures routinely tested negative for mycoplasma contamination.

Subculture passages were detached by enzymatic treatment of tumor cells with trypsin-EDTA solution (0.05%/0.02%; Gibco BRL). Suspended vital cells were counted by methylene blue exclusion. Cells from each third subculture population were cryopreserved at −196°C in DMEM cell culture medium containing 15% (v/v) dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany).

Morphology

Cell culture cells were routinely examined by light-microscopy. Microphotographs were obtained from a Leica invertoscope (Type 090–131.001, Leitz, Wetzlar, Germany). They were documented by an OM-1 camera. Differences in cell morphology, colonization and adherence of the cells were examined.

Chemicals

Commercially available cis-diamminedichloroplatinum (II) (CDDP; Medac, Hamburg, Germany), methotrexate-di-natrium (MTX; Cyanamid-Lederle, Wolfratshausen, Germany), vinblastine-sulfate (VBL; Lilly, Bad Homburg, Germany) and etoposide (VP16; Bristol, Neu-Isenburg, Germany) were provided as sterile solutions. Anticancer agents, that were used for the continual supplementation of culture media, were diluted with physiological saline to final drug concentrations (10^{-10}–10^{-2} mol/l). Those vials were kept as stock solutions at −80°C. In contrast, the drug concentrations required for the determination of the cytotoxicity were always freshly prepared.

Induction of drug-resistant subculture cells

Parental HT1376 and TCCSUP cells were initially exposed to 10^{-10} mol/l cytostatic drugs, that were added to standard cell culture medium. Subsequent cell culture passages were treated with progressively increasing concentrations (10-times) of the drugs whenever 70% of confluent density of exponentially growing tumor cells occured. As many as 50% of the tumor cells lost adherence or died, or if more than 50% of the cells developed significant morphological changes, such as bizarre appearance or giant cytosolic vacuoles, the treatment of the cells with cytostatic agents was discontinued until subcultures reattained a regular 70% cell confluent density of less affected tumor cells. Afterwards the drug exposure was continued with the last tolerated concentration. Cells were subsequently treated by gradually decreasing dosages (≤5-times).

Determination of growth inhibition, quantification of resistance

Relative resistance and cross-resistance were quantified by comparing dose-response curves from different cell lines. Cells were exposed to CDDP (5×10^{-7} to 5×10^{-5} mol/l), MTX (10^{-9} to 5×10^{-5} mol/l), VBL (8.75×10^{-10} to 10^{-8} mol/l) or VP16 (10^{-8} to 5×10^{-4} mol/l) for 72 h.

Growth inhibition was measured by use of a colorimetric cytotoxicity assay (SRB). Details have been described elsewhere [23, 40]. In brief, 10 000 tumor cells were allowed to settle in microtiter plates (Becton Dickinson Labware, N.J.) for 2 h in complete medium, before they were exposed to medium containing anticancer drug for 72 h. Controls received no cytostatic agents. Afterwards cells were fixed with a final concentration of 10% (v/v) trichloroacetic acid (TCA) for 5 min at 4°C. Plates were rinsed with deionized water and dried at room temperature (24 h). Cells were stained with 0.4% (w/v) sulfurhodamine B solution for 10 min. Unbound stain was removed by 10% (v/v) acetic acid. Bound stain was solubilized with TRIS-buffer (TRIS-(hydroxymethyl)-aminomethan; pH 10.5). Optical densities were measured at a single wavelength of 515 nm on an automated spectrophotometric plate reader (EAR 400 AT; SLT Labinstruments, Crailsheim, Germany). Growth inhibtion (GI) of treated tumor cells (T) was compared with untreated control (C) cells (T/C %). The drug concentrations, that resulted in 50% growth inhibition (IC50) or 30% growth inhibition (IC30) were determined from corresponding dose-response curves.

Drug resistance was defined by resistance factors (RF). RF50 was defined as IC50 (drug-resistant cells)/IC50 (parental cells), and RF30 similarly. Results from quadruplicates per drug concentration have been repeated at least once. Moreover, those growth inhibition occured at the peak plasma level concentrations of each drug (GI-PPL), which could clinically be achieved by administration of the drugs as in the CMV protocol, were compared between drug-resistant and parental tumor cells.

Cell cycle distribution by DNA-flowcytometry

DNA-flowcytometry was performed by a modified procedure according to Otto and coworkers [28]. Tumor cells growing in log growth phase were harvested by trypsinization from cell culture flasks. Normal human lymphocytes were used as standard. Vital cells 10^7 were fixed in 5 ml 70% (v/v) alcohol at 4°C for 4 h. The cell pellet (10 min, 1100 g) was resuspended in 1 ml of a solution containing 2.1% (w/v) citric acid and 0.5% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20) for 10 min at room temperature. Afterwards, cells were stained with 0.5% (w/v) 4′, 6-diamidino-2-phenylindol (DAPI; Serva, Heidelberg, Germany). At least 10 000 cells were measured at 360 nm on a FACstar plus flowcytometer (Becton Dickinson, Heidelberg, Germany).

Statistics

Optical densities were statistically compared using the non-paired Wilcoxon test. Statistics were performed on the SAS system (Statistical Analysis System, SAS Institute, Cary, N.C.). A P-value of < 0.05 was designated statistically significant.

Results

Final cell culture conditions of drug-resistant cell lines

Wild-type TCCSUP and HT1376 cells were exposed to gradually increasing concentrations of different chemotherapeutic drugs for 20 months on average. Eight different subculture cell lines were established: HT1376-CDDP, HT1376-MTX, HT1376-VBL, HT1376-VP16, TCCSUP-CDDP, TCCSUP-MTX, TCCSUP-VBL and TCCSUP-VP16. Table 1 summarizes the finally tolerated concentrations of anticancer drugs, that were routinely supplemented to the cell culture media of the different subculture cell lines. Dose-response curves confirmed for all drug-resistant subcultures that no GI occured at those drug concentrations.

Morphology

During the adaptation of parental cells to increasing concentrations of anticancer agents, different signs of cell damage were observed: loss of adherence, cell deformation from a round shape to a polygonal appearance, an increase of tumor cell volume (5–10 times), and the appearance of giant cells or the appearance of large vacuoles and cytoplasmatic granules could be noticed. Drug-resistant subcultures finally grew as anchoring monolayer cultures in vitro. They still contained an increased number of small cytosolic vesicles, compared with the parental cells.

Table 1 Finally tolerated concentrations of anticancer drugs in cell culture medium of drug-resistant cell lines

Cell line	Concentration
HT1376-CDDP	0.5 μmol/l CDDP
HT1376-MTX	1 μmol/l MTX
HT1376-VBL	0.005 μmol/l VBL
HT1376-VP16	0.1 μmol/l VP16
TCCSUP-CDDP	1 μmol/l CDDP
TCCSUP-MTX	0.05 μmol/l MTX
TCCSUP-VBL	0.001 μmol/l VBL
TCCSUP-VP16	1 μmol/l VP16

Growth inhibition of the chemonaive parental cells

With regard to IC50 and IC30 concentrations (Table 2), parental HT1376 cells were 1.7–50 times less sensitive to all investigated chemotherapeutic agents, than wild-type TCCSUP cells. VBL was found to cause growth inhibition of both parental cells at lower molar concentrations than MTX, CDDP, and VP16, respectively. Referring to reported peak plasma level-concentrations (PPL) CDDP, MTX, and VP16 caused almost equal growth inhibition (GI-PPL: 55%–70%), except for HT1376 cells, that were only slightly inhibited by VP-16 (25%). Clinically relevant PPL were chosen with regard to the concentrations of the drugs usually administered in the CMV-schedule (CDDP: 100 mg/m^2; MTX: 30 mg/m^2; VBL: 4 mg/m^2): PPL(CDDP): approximately 3×10^{-6} mol/l, PPL (MTX): approximately 1×10^{-6} mol/l, PPL(VBL): approximately 4×10^{-7} mol/l. PPL(VP16): approximately 3×10^{-6} mol/l was chosen for an oral application of 60 mg/m^2 etoposide.

Resistance of drug-resistant cell lines against the inductive cytostatic drugs

Calculated resistance factors (RF50s and RF30s) are depicted in Table 3: Achieved RF50s of drug resistant subcultures from HT1376 and TCCSUP cells were similar for an exposition with CDDP and VP-16 (~4–5×). In contrast, TCCSUP-MTX subcultures developed approximately twice the resistance against MTX, compared with HT1376-MTX cells. Vice versa, HT1376-VBL cells developed two-fold enhanced resistance against VBL, compared with TCCSUP-VBL cells. Therefore, the initially more drug-sensitive parental TCCSUP cells developed stronger resistances against MTX, since HT1376 cells were more capable of developing resistance against VBL (Table 3). However, all drug-resistant subculture cell lines from TCCSUP (-CDDP, -MTX, -VBL, -VP16) still remained more sensitive to the inductive anticancer agents at equal molar concentrations of the drug, compared with drug-resistant subcultures from HT1376 cells (-CDDP, -MTX, -VBL, -VP16) (Table 2). Interestingly, with re-

Table 2 IC50 value and IC30 concentrations of cisplatin (CDDP), methotrexate (MTX), vinblastine (VBL) and etoposide (VP16) for different drug-resistant cell lines (in μmol/l)

Cell line	CDDP		MTX		VBL		VP16	
	IC50	IC30	IC50	IC30	IC50	IC30	IC50	IC30
HT1376	5	2	0.4	0.1	0.005	0.00375	50	20
HT1376-CDDP	25	12.5	20	5	0.03[a]	0.01	200[a]	50
HT1376-MTX	10	3	90[a]	30	0.0125[a]	0.009	100	50
HT1376-VBL	10	3	40	0.1	0.0375[a]	0.01	60	30
HT1376-VP16	5	2	50	20	0.06[a]	0.02	200[a]	70
TCCSUP	2	0.6	0.1	0.06	0.003	0.0015	2	0.4
TCCSUP-CDDP	7.5[a]	4	0.4	0.09	0.003	0.0015	2	0.8
TCCSUP-MTX	2.5	1	50[a]	0.5	0.004	0.0025	7	1
TCCSUP-VBL	4	2	30	20	0.009	0.0025	10	5
TCCSUP-VP16	3.5	2	25	1	0.004	0.0025	10	5

[a] Derived by extrapolation of graphs

gard to the different anticancer drugs, MTX led to much stronger resistances (HT1376-MTX (Fig. 1) and TCCSUP-MTX subcultures: RFs > 200), than the other drugs (RFs < 10) (Table 3).

Cross-resistance of drug-resistant cell lines

Pleiotropic relative cross-resistances were observed for six of eight drug-resistant subculture cell lines (75%). Corresponding RF50s and RF30s are shown in Table 3. Notably, TCCSUP-VBL cells developed relative cross-resistance against MTX (Fig. 2) ≫ VP16. TCCSUP-VP16 subcultures also gained cross-resistance against MTX. Mutual induction of relative cross-resistance was not detected for TCCSUP subcultures at all.

In contrast, HT1376-CDDP cells exhibited cross-resistance against MTX ≫ VBL > VP16, HT1376-MTX against VBL ≥ VP16, HT1376-VBL against MTX ≫ CDDP and HT1376-VP16 against MTX ≫ VBL (Fig. 3). Overall, the drug-resistant subculture cell lines from the intrinsically more drug-insensitive parental HT1376 cells developed cross-resistances three-times more often (9 out of a maximum of 12 possible; 75%), than subcultures from the initially more drug-sensitive parental TCCSUP-cells (3/12; 25%). Interestingly, all subcultures from HT1376 cells achieved significant relative cross-resistances against MTX and VBL, since only HT1376-CDDP and HT1376-MTX subcultures showed relative cross-resistance against VP16. Moreover, only HT1376-VBL subcultures developed relative cross-resistance against CDDP (Table 3). Mutual induction of

Table 3 Resistances and cross-resistances of drug-resistant subculture cell lines from HT1376 and TCCSUP cells against cytostatic agents. For definition of resistance factors RF50 and RF30: see text. Only significant data are depicted. Significance ($P < 0.05$) was calculated with regard to the parental cell line; -: not significant. For legend see Table 2

Cell line	CDDP		MTX		VBL		VP16	
	RF50	RF30	RF50	RF30	RF50	RF30	RF50	RF30
HT1376-CDDP	5	6.25	50	50	6	2.7	4	–
HT1376-MTX	–	–	225	300	2.5	2.4	2	–
HT1376-VBL	2	–	100	–	7.5	2.7	–	–
HT1376-VP16	–	–	125	200	12	5.3	4	3.5
TCCSUP-CDDP	3.75	6.7	–	–	–	–	–	–
TCCSUP-MTX	–	–	500	8.3	–	–	–	–
TCCSUP-VBL	–	–	300	333	3	–	5	12.5
TCCSUP-VP16	–	–	250	16.7	–	–	5	12.5

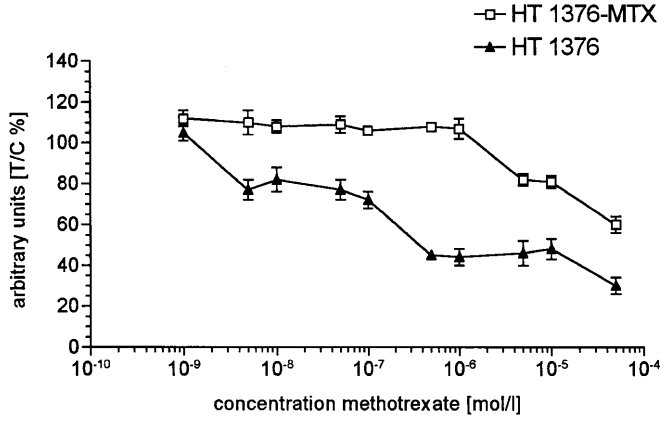


Fig. 1 Dose-response curves of HT1376 and HT1376-MTX cells for treatment with methotrexate

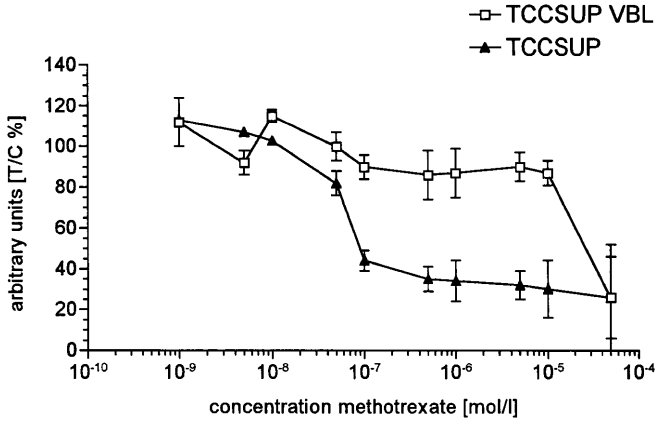
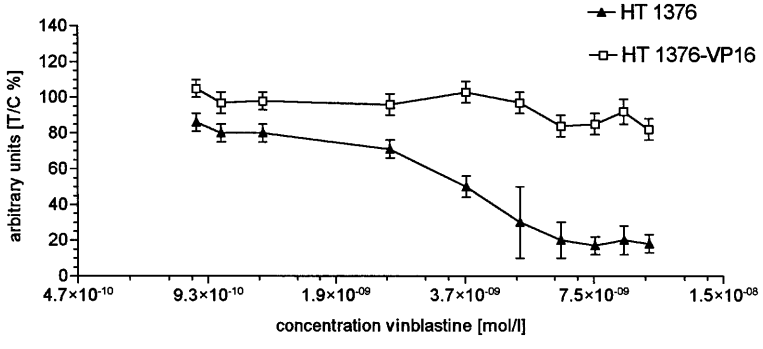


Fig. 2 Dose-response curves of HT1376 and TCCSUP-VBL cells for treatment with methotrexate

Fig. 3 Dose-response curves of HT1376 and HT1376-VP16 cells for treatment with vinblastine



relative cross-resistances was found for HT1376 subcultures between CDDP and VBL, MTX and VBL, and MTX and VP16, but not between CDDP and MTX or CDDP and VP16. Overall, highest RF50 (50–500×) and most cross-resistances (five of six cell lines) occured for treatment with MTX, since cross-resistance for CDDP was found in only one of six cell lines.

Flow-cytometry

HT1376 cells were characterized by an elevated DNA content compared with TCCSUP cells. No significant changes in DNA index (DI) were found in any drug-resistant subculture compared with the parental cells. S-phase fractions and the G2/M-phase fractions of the different drug-resistant sublines are summarized in Table 4. The S-phase fraction was increased for HT1376-subcultures, except for HT1376-MTX cells. In contrast, the G2/M-phase fraction was markedly increased for all drug-resistant TCCSUP-subcultures.

Discussion

Polychemotherapy of advanced or metastasized transitional cell carcinoma with CMV led to complete response rates in excess of 20%. Tumor progression or relapse and the failure to respond to further chemotherapy are suggested to be due to intrinsic or acquired drug resistance of tumor cell populations. Conclusively it has been demonstrated that in transitional cell cancer, the multidrug resistance phenotype is increased in tumors from patients treated by chemotherapy, compared with untreated primary lesions [4, 30].

Only a few drug-resistant cell lines from human TCC have been reported in the past ($n = 14$). Cell lines have usually been selected for resistance by continuous or intermittent exposure of chemonaive parental cells to gradually increasing or constant concentrations of one single chemotherapeutic agent. Those studies provide strong support for the premise that resistance to anticancer drugs is multifactorial. Table 5 summarizes different pathways that have been suggested to cause drug resistance in bladder carcinoma cell lines; Table 6 reviews their resistance pattern. Of interest is that MGH-U1 cells, as well as some cultures of J82 cells, are in fact subcultures derived from parental T24 cells [26], thus as many as 9 of 14 established drug-resistant cell lines are of the same origin.

Most drug-resistant bladder carcinoma cell lines were selected for resistance against doxorubicin [10, 13, 16, 19, 20, 24, 35, 37]. Resistance for this drug and cross-resistance for etoposide, vinblastine and epidoxorubincine was most likely to result from classic MDR-1 gene product, P170-glycoprotein overexpression. However, resistance was also related to the multidrug resistance-associated protein (MRP) [13, 16], resulting in cross-resistance to cisplatin or methotrexate. Besides an overexpression of the MRP gene, decreased cellular level of DNA topoisomerase II were suggested to be responsible for atypical drug resistance against vinca alkaloids and etoposide [24]. Interestingly, T24/ADM-9 and KK47/ADM cells were characterized by the appearance of morphologically bizarre-shaped cells, and weaker cell-to-cell attachment [16, 24]. Similar phenomena have been recognized for most of the present cell lines during their process of adaptation to anticancer agents. Drug-resistant tumor cells may contain an increased number of intracytoplasmic vesicles due to the attempt of the cell to exclude the drugs by enhanced vesicular transportation mechanisms [36]. However, the frequent persistence of giant cells, observed in MGH-U1R cells [20], was not evident in our drug-resistant subculture cell lines. Less frequently, there have been reports about bladder cancer cell lines with defined resistance against mitomycin-C [8, 38, 39, 47]. To our knowledge, no report exists about MTX-, VBL- or VP16 resistant cell lines from human TCCB, and only two cell lines have already been selected for resistance against cisplatin [22, 45].

Therefore, the present study aimed to establish new drug-resistant subculture cell lines from human bladder cancer cell lines. Care was taken to select two cell lines that were derived from chemonaive patients: HT1376 (derived from a patient with a bladder cancer T2 G3 M0) [31] and TCCSUP (derived from an untreated patient with a bladder carcinoma T4 G4 M1) [25]. The experiments focused especially on the activity of the single anticancer agents that are administered in the

Table 4 Cell cycle distribution of exponentially growing tumor cells

Cells	DNA-index	G_0G_1 fraction (%)[a]	S-phase fraction (%)[a]	G_2/M-phase fraction (%)[a]
HT1376	2.4	58.3	19.9	21.8
HT1376-CDDP	2.28	46.8	31.9	21.3
HT1376-MTX	2.27	70.4	11.3	18.3
HT1376-VBL	2.19	62.2	24.5	13.3
HT1376-VP16	2.5	51.9	29.7	18.4
TCCSUP	1.5	61.9	32.4	5.7
TCCSUP-CDDP	1.5	52	28.1	19.9
TCCSUP-MTX	1.49	46.9	33.8	19.3
TCCSUP-VBL	1.47	47.1	32.5	20.4
TCCSUP-VP16	1.59	58.6	25.3	16.1

[a] % = mean of two different experiments
The DNA-index (DI) was defined as the equation of ploidy of tumor cells/ploidy of normal human leukocytes [9]

Table 5 Bladder carcinoma cell lines selected for anticancer drug resistance (review of the literature)

Drug-resistant cell line	Parental cell line	Wild-type classification	Resistance mechanisms	Reference
			For cisplatin:	
RT112-CP	RT112	Tx G3; CN	Glutathione reductase ↑ glutathione peroxidase ↑	[3] [45]
T24/CDDP	T24	T3 G3; CN	Activated oncogene (c-*myc*)	[22]
			For vinca alkaloids:	
J82/NVB	T24	T3 G3; CN	Microtubule dynamics	[7]
T24/VCR	T24	T3 G3; CN	MDR1 mRNA ↑ (DNA topoisomerase II ↓)	[13]
			For doxorubicine:	
MGH-U1R	T24	T3 G3; CN	mdr-1 gene ; P170-glycoprotein	[10, 19, 20]
KK47/ADM	KK47	Ta G1; CN	P-glycoprotein ↑ MDR1 mRNA ↑, MDR1 DNA ↑ MRP mRNA ↑ (DNA topoisomerase II ↓)	[13, 16]
UM-UC-6dox	UM-UC-6	Tx Gx	P170-glycoprotein ↑	[37]
T24/ADM-1	T24	T3 G3; CN	MRP mRNA ↑, MRP DNA ↑ DNA topoisomerase II ↓	[13]
T24/ADM-2	T24	T3 G3; CN	MRP mRNA ↑, MRP DNA ↑ DNA topoisomerase II ↓	[13]
T24/ADM-9	T24	T3 G3; CN	MRP mRNA ↑ DNA topoisomerase II ↓	[24]
RT112/D21	RT112	Tx G3; CN	P-glycoprotein ↑	[35]
			For mitomycin-C:	
RT112-MMC	RT112	Tx G3; CN	NAD(P)H: Quinon Oxidoreductase↓ Glutathione transferase↓	[8]
J82/MMC	T24	T3 G3; CN	DT-diaphorase ↓ Cytochrome P450 reductase ↓ Glutathione transferase ↑ Glutathione reductase ↓ DNA ligase I mRNA ↓ Catalase ↑	[47] [39]
J82/MMC2	T24	T3 G3; CN	DT-diaphorase ↓↓ Cytochrome P450 reductase ↓ Glutathione transferase ↑ DNA ligase I mRNA ↓ DNA polymerase β ↑	[39]

CN chemonaive, *T* tumor stage, *G* tumor grade

CMV polychemotherapy protocol (**C**DDP, **M**TX, **V**BL) [15, 49], which is broadly accepted as the chemotherapeutic regimen in patients with advanced or metastatic bladder carcinoma. Finally, eight subculture cell lines, selected for the present experiments, persisted to divide and multiply in the continual presence of up to 1 μmol/l anticancer agent. All drug-resistant subculture cells maintained a slower growth rate than the parental cells, as it has already been shown for doxorubicin-resistant MGH-U1R, RT112/D21 and KK47/ADM cells [16, 20, 35].

Referring to peak-plasma concentrations, CDDP and MTX gave a similarly strong growth inhibition of HT1376 and TCCSUP cells. This result reflected clinical experience, in that monochemotherapy with CDDP and MTX led to comparable objective remission rates (approximately 30%) [48]. Parental HT1376 cells showed an elevated intrinsic resistance to all investigated chemotherapeutic agents, compared with poorly differentiated parental TCCSUP cells. These data may confirm, that de-differentiated transitional cell carcinoma cells show a reduced level of multidrug resistance-associated protein (MRP) [6, 13]. Moreover, the increased intrinsic resistance of HT1376 cells against the alkylating drug cisplatin may partly be due to overexpression of inactivated p53 protein, whereas TCCSUP cells do not harbor mutations of wild-type p53 [11]. Inactivation of p53 has been suggested to inhibit apoptosis after DNA damage by anticancer agents, leading to drug resistance. Since p53-deficient tumor cells have an incompetent G1 checkpoint of the cell cycle, they accumulate at the G2 checkpoint, preventing replication of damaged DNA and allowing time for repair [51]. Consistent with this hypothesis, the observed, basically increased number of parental HT1376 cells at the G2/M transition of the cell cycle, compared with TCCSUP cells, is likely to explain the higher intrinsic drug resistance of HT1376 against CDDP.

At the least, all eight established subculture cell lines expressed significant resistances against the inductive chemotherapeutic drugs. Despite strong activity of MTX against both chemonaive parental cell lines, the continuous exposition with the drug led to an extraordinarily high rate of resistance (225 to >500 times) at

Table 6 Comparision of resistance factors (RFs) from different multidrug-resistant cell lines from human urothelial carcinoma, compared with the present cell lines. All cell lines were derived from chemonaive parental cell lines. Resistance factors are usually referring to IC50 (except for T24/CDDP cells). *No c-r* no cross-resistance, – not done, *MTT*/*SRB* colorimetric assays, *CFA* colony-forming assay

Cell lines selected for	CDDP	MTX	VBL	DOX (RFs)	VP16	VM26	MMC	Assay	Reference
Cisplatin									
RT112-CP	10	6.6	–	No c-r	1.8	10	–	MTT	[45]
T24/CDDP	2.3–3.6	–	–	–	–	–	–	MTT	[22]
HT1376-CDDP	5	50	6	–	4	–	–	SRB	
TCCSUP-CDDP	3.75	No c-r	No c-r	–	No c-r	–	–	SRB	
Methotrexate									
HT1376-MTX	No c-r	225	2.5	–	2	–	–	SRB	
TCCSUP-MTX	No c-r	500	no c-r	–	no c-r	–	–	SRB	
Vinca alkaloid									
J82/NVB	–	No c-r	16	1.5	1.4	nd	nd	Count	[7]
T24/VCR	2.2	–	–	1.8	6.8	–	–	CFA	[13]
HT1376-VBL	2	100	7.5	–	No c-r	–	–	SRB	
TCCSUP-VBL	No c-r	300	3	–	5	–	–	SRB	
Doxorubicin									
MGH-U1R	–	–	188	40	13	–	–	CFA	[10]
KK47/ADM	No c-r	No c-r	150	271	25	No c-r	No cr	count	[16]
	No c-r	–	–	18.7	3.5	–	–	CFA	[13]
UM-UC-6dox	–	–	–	6.6	1.9	–	–	MTT	[37]
T24/ADM-1	No c-r	–	–	4.8	5.1	–	–	CFA	[13]
T24/ADM-2	No c-r	–	–	9.3	10.5	–	–	CFA	[13]
T24/ADM-9	No c-r	–	6.6	9.2	3.7	–	No c-r	MTT	[24]
RT112/D21	No c-r	No c-r	168	96	–	–	No c-r	MTT	[35]
Etoposide									
HT1376-VP16	No c-r	125	12	–	4	–	–	SRB	
TCCSUP-VP16	No c-r	250	No c-r	–	5	–	–	SRB	
Mitomycin									
RT112-MMC	–	–	–	–	–	–	40	SRB	[8]
J82/MMC	2	–	–	No c-r	No c-r	3	6	CFA	[47]
J82/MMC2	2	–	–	–	No c-r	–	9.6	CFA	[39]

HT1376-MTX and TCCSUP-MTX cells, and five of six (83%) subculture cell lines, with drug resistances against other anticancer inducing agents, also developed high cross-resistance to MTX (50–300 times). Resistance to MTX is thought to result from an increased expression of the target enzyme dihydrofolate-reductase, a decreased affinity of dihydrofolate-reductase to MTX [12, 32] or an insufficient drug uptake by the folate/MTX transporter. Interestingly, the cisplatin resistant bladder carcinoma cell line RT112-CP also developed cross-resistance to MTX [45]. A similar observation has been made for cisplatin-resistant cervical carcinoma cells, that exhibited cross-resistance against MTX and vincristine [27]. However, because MTX does not share structural or functional similarities with CDDP, VBL or VP16, the underlying mechanism for the observed high cross-resistance against MTX in our experiments is crucial if there are single specific resistance mechanisms, rather than a multifocal basis for resistance. Nevertheless, the clinically achieved response rate after monochemotherapy with MTX of 29% [49], as well as our present data on the activity of MTX at peak plasma level concentrations in vitro, may argue against the impression that the high capacity of MTX to induce resistance in transitional cell carcinoma in vitro necessarily means unresponsiveness. But the observed high rate of drug resistance against MTX in vitro may argue against frequent, repeated application of a polychemotherapy regimen containing MTX, especially in cases of progressive disease or tumor relapse after previous therapy with CMV.

In contrast to this, the central role of cisplatin for treatment of patients with advanced or metastatic bladder carcinoma was confirmed by the present in vitro experiments: CDDP resulted in strong growth inhibition of most drug-resistant subculture cell lines and both parental cell lines. Moreover, resistance against CDDP was moderate, and only HT1376-CDDP cells exhibited cross-resistances to other drugs. Similar to our observation, cross-resistance against MTX and VP-16 has also been reported for RT112-CP cells [45]. The overexpression of repair enzymes such as alkyltransferases serves as drug resistance mechanisms for the alkylating agent cisplatin [1, 3, 21]. Since etoposide and cisplatin both inhibit topoisomerase II, decreased content of active topoisomerase II transcripts [42] may have driven cross-resistance of HT1376-CDDP cells for VP16. Moreover it has been shown, that cell lines that have been selected for topoisomerase inhibitors developed relative cross-resistance against different cytostatic drugs

independent from p170-gp expression (atypical multidrug resistance, at-mdr) [2], as it might have occured for the cross-resistances of HT1376-CDDP cells against MTX or VBL. Finally, it has been shown for the human prostate cancer cell line PC3, that cross-resistance between cisplatin and etoposide can result from high expression of certain proto-oncogenes [50]. In fact, the resistance of T24/CDDP cells [22] against cisplatin could be overcome by treatment with c-*myc* antisense oligonucleotides, indicating the influence of activated oncogenes. Consistent with this, it has more recently been confirmed by transformation of keratinocytes that pleiotropism in drug resistance against anticancer agents, which do not share structural or functional similarities (e.g. cisplatin, doxorubicin, vincristine), could be induced by an oncogene [33].

Drug-resistant bladder carcinoma cell lines selected for vinblastine resistance, such as HT1376-VBL and TCCSUP-VBL, have not been described before. The observed relative cross-resistance of HT1376-VP16 cells against vinblastine and TCCSUP-VBL cells against etoposide are most likely based on an overexpression of p170-glycoprotein [37, 46]. In this context it has been shown that normal urothelial cells do not express p170-gp, since TCCB overexpresses p170-gp, especially after previous chemotherapy [4].

In our experiments, VP16 caused lower growth inhibition of HT1376 cells (with regard to GI-PPL) than CDDP and MTX. Therefore, the activity of the drug on both cell lines tended to reflect the lower response rate of patients with chemonaive TCCB to monochemotherapy with etoposide (OR: 20%) [29]. However, VP16 revealed only a small potential to induce resistance and, especially, induced no cross-resistance to CDDP. Moreover, since VBL- and CDDP-resistant cell lines did exhibit much smaller cross-resistance to VP16 than to MTX, these observations suggest that a chemotherapy regimen combining VP16 with CDDP and VBL can be useful for the clinical management for chemonaive or pre-treated human bladder carcinoma.

In conclusion, the present study reports on a panel of eight bladder carcinoma cell lines with reproducible drug-resistance against the single anticancer drugs of the CMV-polychemotherapy protocol and against etoposide. The high rate of relative cross-resistance to structually unrelated agents and the heterogeneous pattern of resistance against the inductive agents, alluded to a multifocal basis for resistance. The observed pleiotropism indicates concomitant induction of different pathways for drug resistance in human bladder carcinoma cell lines in vitro. Therefore, the established drug-resistant subculture cell lines provide experimental systems for further investigations about multidrug resistance of bladder carcinoma cell lines. They serve as an appropriate tool to screen new agents, which will arise in the treatment of patients with advanced or metastasized TCCB in the future, or to develop strategies for the sensitization of drug-resistant bladder cancers to chemotherapeutic drugs. The cell lines are also useful for studying the various mechanisms underlying drug-resistance, although stability of resistance in the absence of the inductive drugs remains to be clarified. The clinical implication of the observation that urothelial carcinoma cells are capable of developing pleiotropic resistances against the single drugs of the CMV-polychemotherapy protocol, is likely to suggest that the administration of cisplatin in polychemotherapeutic approaches against urothelial carcinoma is essential, since repetetive application of MTX appeared to be doubtful.

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