Human bladder cancer cells undergo cisplatin-induced apoptosis that is associated with p53-dependent and p53-independent responses

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Abstract. Cisplatin is a first-line chemotherapeutic agent and a powerful component of standard treatment regimens for several human malignancies including bladder cancer. DNA-Pt adducts produced by cisplatin are mainly responsible for cellular toxicity and induction of apoptosis. Identification of the mechanisms that control sensitivity to cisplatin is central to improving its therapeutic index and to successfully encountering the acquired resistance frequently emerging during therapy. In the present study, using MTT-based assays, Western blotting and semi-quantitative RT-PCR, we examined the apoptosis-related cellular responses to cisplatin exposure in two human urinary bladder cancer cell lines characterized by different malignancy grade and p53 genetic status. Both RT4 (grade I; wild-type p53) and T24 (grade III; mutant p53) cell types proved to be vulnerable to cisplatin apoptotic activity, albeit in a grade-dependent and drug dose-specific manner, as demonstrated by the proteolytic processing profiles of Caspase-8, Caspase-9, Caspase-3, and the Caspase repertoire characteristic substrates PARP and Lamin A/C, as well. The differential resistance of RT4 and T24 cells to cisplatin-induced apoptosis was associated with an RT4-specific phosphorylation (Ser15; Ser392) pattern of p53, together with structural amputations of the Akt and XIAP anti-apoptotic regulators. Furthermore, cisplatin administration resulted in a Granzyme B-mediated proteolytic cleavage of Hsp90 molecular chaperone, exclusively occurring in RT4 cells. To generate functional networks, expression analysis of a number of genes, including Bik, Bim, Bcl-2, FAP-I, Fas, Fasl, TRAIL, Puma, Caspase-10, ATP7A, ATP7B and MRP1, was performed, strongly supporting the role of p53-dependent and p53-independent transcriptional responses in cisplatin-induced apoptosis of bladder cancer cells.

Introduction

With its incidence continuing to increase, bladder cancer is classified among the five most common malignancies in industrialized countries (1). Forming a worldwide estimate over one million patients, bladder cancer is clearly considered a significant public health issue around the world (2). The three main types of cancer affecting bladder are transitional cell carcinoma (TCC), squamous cell carcinoma (SCC) and adenocarcinoma (ADC), with TCC representing >90% of all bladder cancers. Four clinically distinct entities of TCC have been recognized: superficial, papillary tumors (Ta and T1), carcinoma in situ (Tis), muscle-invasive tumors (T2-T4) and advanced disease, involving extra-pelvic nodal or distant metastasis (3,4). Metastatic TCC demonstrates a moderate sensitivity to chemotherapy while a significant variation in patient survival rates and activity levels of individual regimens has been observed (5).

Cisplatin-based combination chemotherapy protocols, such as MVAC (methotrexate-vinblastine-adriamycin-cisplatin), constituted for a number of years standard treatment of patients with advanced (or metastatic) urothelial bladder cancer. However, due to the MVAC-induced systemic toxicity, novel therapeutic schemes containing different drug-coctails have been developed, with cisplatin occupying a central position...
The platinum atom of cisplatin forms covalent bonds that DNA is the preferential and cytotoxic target for cisplatin proteins, RNA and DNA. However, it is generally accepted that the derivative can subsequently interact with a variety of cellular components bearing nucleophilic sites, including, among others, cytoskeletal microfilaments, membrane phospholipids, proteins, RNA and DNA. However, it is generally accepted that DNA is the preferential and cytotoxic target for cisplatin (7-9). The platinum atom of cisplatin forms covalent bonds to the N-7 atoms of the imidazole rings of guanosine and adenosine that reside in the major groove of the double helix, thus resulting in the formation of DNA adducts. These are typified in six major categories characterized by 1,2-d(GpG) intrastrand crosslinks, 1,2-d(ApG) intrastrand crosslinks, G-G interstrand crosslinks, monofunctional crosslinks and intermolecular DNA-protein crosslinks, all inducing prominent, albeit variable, DNA structural distortions (6-10).

Cisplatin-mediated damage of genomic DNA causes severe cell cycle perturbation and arrest at certain checkpoints, while in the absence of adequate repair the affected cells undergo programmed cell death via an apoptosis process. The specific apparatus that triggers apoptosis in response to cisplatin should not only be able to detect genome detriments, but also to determine whether damage is sufficiently severe to be lethal (6,7,9-12). Transduction of DNA-damage signals induced by cisplatin critically modulates, among others, the apoptosis-promoting activity of p53 transcription factor and the cell survival–protecting function of Akt serine/threonine kinase (6,9,13-19). Interestingly, mutations in the p53 genetic locus have been strongly implicated in bladder cancer development, whereas there has been controversy regarding the role of p53 in cellular resistance to cisplatin (3,4,6,20). The relationship between p53 status and cisplatin cytotoxicity should depend on tumor cell type, activation of specific signaling pathways, the presence of additional genetic alterations and other p53-independent determinants (6).

In this context, here, we have studied the cisplatin-induced apoptotic responses of two human urinary bladder cancer cell lines, mainly distinguished by their disparate grade of malignancy. Our data demonstrate that the differential resistance of the p53-proficient RT4 cell line (grade I) and p53-deficient T24 cell line (grade III) to cisplatin-mediated apoptosis is tightly associated with p53-dependent and p53-independent cellular activities. The critical role of p53, Akt, XIAP and Hsp90 proteins was evaluated, while the expression profile of several genes involved in apoptosis and resistance to the drug was also examined.

**Materials and methods**

**Drugs and reagents.** Cisplatin was purchased from EBEWE Pharma Ges.m.b.H. Nfg.KG (Unterach, Austria). The antibodies recognizing Caspase-3 (#9662), Caspase-8 (#9746), Caspase-9 (#9502), PARP (#9542), Lamin A/C (#2032), β-Actin (#4968), p53 (#9282), Phospho-p53-Ser15 (#9284), Phospho-p53-Ser392 (#9281), Akt (#9272), Phospho-Akt-Ser473 (#9271), XIAP (#2042) and Granzyme B (#4275) were obtained from Cell Signaling Technology Inc. (Hertfordshire, UK), whereas the antibody against Hsp90 (SC-13119) was purchased from Santa Cruz Biotechnology Inc. (California, USA). ECL Western blots reagents were supplied by GE Healthcare-Amersham (Buckinghamshire, UK). Gene-specific oligonucleotide primers were synthesized by Metabion (Martinsried, Germany) and Operon (California, USA). All the other chemicals were obtained from Sigma-Aldrich (MO, USA), Fluka (Hannover, Germany) and AppliChem GmbH (Darmstadt, Germany).

**Cells and culture conditions.** The established cell lines from TCC human bladder cancer, RT4 (grade I) and T24 (grade III) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and Professor John R. Masters (Prostate Cancer Research Centre, Institute of Urology, UCL, UK), respectively. Both cell lines were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 50 mM sodium bicarbonate and 1x non-essential amino acids at 37°C and 5% CO2. Contrary to the wild-type p53 genotype of RT4 cells, the T24 cell line contains a p53 mutant allele that carries an in-frame deletion of the TAC triplet encoding tyrosine 126 (V126) (21,22). All cell culture media and reagents were purchased from Biochrom AG (Berlin, Germany).

**Cell viability assay.** RT4 and T24 cells were seeded at densities of 20000 cells/well into 48-well flat-bottomed plates, treated with or without cisplatin (1, 10 and 50 μg/ml) and incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cell viability was assessed by MTT assay (23).

**Western blotting.** At 24 h after cisplatin treatment (1 and 10 μg/ml), the RT4 and T24 cells were harvested, washed twice with PBS and solubilized in lysis buffer (15 mM Tris-base, 75 mM NaCl, 5 mM Na3EDTA, 50 mM Na2PO4, 50 mM NaF, 0.5 mM Na4P2O7, 2% Triton X-100, 10% glycerol and 2 mM PMSF (pH 7.6 at 4°C)). After centrifugation at 12000 g for 20 min at 4°C, the supernatant was collected as the total protein extract and stored at -30°C. Protein concentrations were measured using the Bradford-type protein assay (Bio-Rad, USA). Equal amounts of protein (40 μg) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane (Schleicher and Schuell GmbH, Germany). Each membrane was blocked with 5% non-fat dry milk (or 5% BSA where required) in TBS-T [20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.2% Tween-20] for 2 h at room temperature and subsequently incubated with primary antibody (1:1500) for 16 h at 4°C.
Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary antibody (1:2000) and ECL reagents (24). Data were obtained from three different experiments, one of which is illustrated in the present study.

Semi-quantitative RT-PCR analysis. RT4 and T24 cells were treated with the indicated doses of cisplatin for 24 h (0, 1 and 10 \( \mu \)g/ml) and total RNA was extracted with the TRIzol reagent, according to manufacturer’s instructions (Invitrogen, USA). One microgram of RNA was reverse transcribed in 20-\( \mu \)l reaction volume containing an oligo-d(T) primer (Invitrogen) and the MMLV reverse transcriptase (Invitrogen). The resulting cDNA was subsequently amplified by semi-quantitative PCR with the Biometra T3000 thermocycler (Goettingen, Germany), using 10 pmole of each forward and reverse cDNA-specific primer in a final volume of 25 \( \mu \)l. The PCR products were separated by agarose gel electrophoresis following standard procedures (25). The gene-specific names, the forward (F) and reverse (R) primer sequences, the molecular size of the amplified fragments, the annealing temperatures (Ta) and the number of cycles performed for all the examined genes are summarized in Table I. Ampli-

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
<th>Length of PCR product (bp)</th>
<th>Temperature (°C)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Bik</strong></td>
<td>F: 5’-CTG TAT GAG CAG CTC CTG G-3’ R: 5’-GAT GTC CTC AGT CTG GTC G-3’</td>
<td>252</td>
<td>55</td>
<td>30</td>
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<tr>
<td><strong>2. Bim</strong></td>
<td>F: 5’-TGT GAC AAA TCA ACA AAA ACC C-3’ R: 5’-AGT CGT AAG ATA ACC ATT CTG G-3’</td>
<td>230</td>
<td>57</td>
<td>30</td>
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<tr>
<td><strong>3. Bcl-2</strong></td>
<td>F: 5’-TGT GGC CTT CTT TGA GTT CG-3’ R: 5’-AGA GTC AGA TCT CAG TGG AG-3’</td>
<td>222</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td><strong>4. FAP-1</strong></td>
<td>F: 5’-CAG ATG GAA GTA TAG CCT TG-3’ R: 5’-TAG ATG ATC TTT CAC TGG AG-3’</td>
<td>228</td>
<td>55</td>
<td>30</td>
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<td><strong>5. GAPDH</strong></td>
<td>F: 5’-TGG TAT CTG GGA AGG ACT CAT GAC-3’ R: 5’-ATG CCA GTG AGC TTC CCG TTC AGC-3’</td>
<td>189</td>
<td>55</td>
<td>28</td>
</tr>
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<td><strong>6. Fas</strong></td>
<td>F: 5’-GGA TGA ACC AGA CTG CTT GCT G-3’ R: 5’-CTG CAT GTT TTC TGT ACT TCC-3’</td>
<td>371</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td><strong>7. FasL</strong></td>
<td>F: 5’-CTG TGG AAT GGG AAG AAC ACC C-3’ R: 5’-ACC AGA GAG AGC TCA AGC GAG-3’</td>
<td>325</td>
<td>57</td>
<td>40</td>
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<tr>
<td><strong>8. TRAIL</strong></td>
<td>F: 5’-TGG GTA TGA TGG AGG TTC AG-3’ R: 5’-GTG GCC ACT TGA CTT GGC AG-3’</td>
<td>249</td>
<td>57</td>
<td>30</td>
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<tr>
<td><strong>9. Puma</strong></td>
<td>F: 5’-CAG ACT GTG AAT CCT GTG CT-3’ R: 5’-ACA GTA TCT TAC AGG CTG GG-3’</td>
<td>251</td>
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<td>35</td>
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<tr>
<td><strong>10. Caspase-10</strong></td>
<td>F: 5’-CAT AGG ATT GTG CCC ACA CA-3’ R: 5’-CTG GAC ACT CGG CTT CC-3’</td>
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<td>55</td>
<td>32</td>
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<td><strong>11. ATP7A</strong></td>
<td>F: 5’-GCC TCT TGT GAC ACA AGA AAC TG-3’ R: 5’-GCT ATT ACT ACC AAC GCC TC-3’</td>
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<td><strong>12. ATP7B</strong></td>
<td>F: 5’-GCT GAT TGG AAA CCG TGA GT-3’ R: 5’-ATT GCC ATC ATC CCA CAG AG-3’</td>
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<td><strong>13. MRPI</strong></td>
<td>F: 5’-ACC TGG AAA CGG ACC ACC T-3’ R: 5’-TAC TCC TGG ATT TCT CCT TTG-3’</td>
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<td>32</td>
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<tr>
<td><strong>14. CTR1</strong></td>
<td>F: 5’-CCT TCT CAC CAT CAC CCA AC-3’ R: 5’-CTT GTG ACT TAC GCA GCA GC-3’</td>
<td>238</td>
<td>55</td>
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Results

Cytotoxic effects of cisplatin against bladder cancer cells. The cytotoxic activity of cisplatin on the survival profiles of RT4 and T24 cell lines was determined by MTT assay (23). Treatment with cisplatin for 24 h markedly decreased the viability of RT4 and T24 cells in a dose-dependent manner. The survival rates of RT4 cells in the presence of 1, 10 and 50 μg/ml cisplatin were averaged 75.4, 15.23 and 5.41%, respectively. T24 cells demonstrated significant resistance to drug administration, as clearly documented by the 99.4, 59.47 and 37.9% corresponding (mean) survival percentages (Fig. 1). The grade-specific response to cisplatin likely reflects the distinct genetic background of RT4 and T24 cells (i.e., p53 status) and their ability to differentially regulate the cell death process.

Grade-dependent apoptosis induced by cisplatin. Due to the strong cytotoxicity of 50 μg/ml cisplatin when administered to RT4 cells (Fig. 1), severely affecting protein and RNA extract yield, and in an effort to adopt clinically relevant drug doses, all the following treatments were carried out using 1 and 10 μg/ml of cisplatin concentration. After drug administration in both RT4 and T24 cells, the three major apoptotic components Caspase-8, Caspase-9 and Caspase-3 were specifically processed, resulting in the formation of active proteases, howbeit exempting Caspase-8 in T24, as clearly revealed by a Western blotting approach. A similar cleavage profile was detected for PARP and Lamin A/C nuclear proteins, which represent characteristic substrates of the mature Caspase repertoire (Fig. 2). The extent of apoptotic response of different cell types to various drug concentrations could be reliably assessed by a quantified proportion of cleaved [C; (i.e., 17 and 19 kDa for Caspase-3)] to precursor [P; (i.e., 35 kDa for Caspase-3)] protein forms. Therefore, by comparing the C/P respective ratios of RT4 and T24, for all the examined apoptotic mediators, it is unambiguously concluded that T24 are significantly more resistant to cisplatin-induced apoptosis than RT4 cells. Even though the higher drug dose (10 μg/ml) is the effective one regarding proteolytic processing of all the examined proteins, Caspase-8 is the only component being cleaved at the lower cisplatin concentration (1 μg/ml) exclusively in RT4 cells, thus corroborating their enhanced sensitivity to the drug. Moreover, the absence of the Caspase-8 18 kDa fragment in T24, but not in RT4 cells, treated with 10 μg/ml cisplatin (Fig. 2 and data not shown) likely reflects a T24-specific hindrance in the formation of a mature protease and an activation impairment of the cognate membrane death receptor-mediated apoptotic pathway (12,26).

Cisplatin-mediated induction and phosphorylation of p53. Since p53 protein constitutes one of the most critical regulators of DNA damage-induced apoptosis and is frequently associated with chemoresistance in human cancers (6,13-15,18), we sought to evaluate the role of p53 in cisplatin-mediated apoptosis of bladder cancer cells. Lysates derived from control and cisplatin-treated RT4 and T24 cells were subjected to Western blotting, using specific antibodies against total and phosphorylated p53. As shown in Fig. 3, administration of 1 and 10 μg/ml cisplatin in RT4 cells resulted in a strong induction of total and phosphorylated p53 protein levels. Serine residues at positions 15 and 392 (Ser15 and Ser392) were effectively phosphorylated in an RT4-specific manner, thus supporting a role of wild-type p53 in cisplatin cytotoxicity against bladder cancer. Moreover, the higher drug dose (10 μg/ml) appeared to enhance p53 protein instability, as documented by the formation of low molecular weight cleaved forms. In contrast, T24 cells remained almost unresponsive to cisplatin treatment, with an extremely weak upregulation of p53 phosphorylation at Ser15 and an undetectable profile of Ser392 phosphorylation induction and total protein level increase (Fig. 3). In toto, the obtained RT4 and T24 distinct patterns strongly indicate the essential role of Y126 in p53 protein (the missing tyrosine residue in T24 cells), regarding its activity and function under DNA damaging conditions (21,22).
Deregulation of Akt and XIAP signaling integrity by cisplatin.

It has been previously suggested that alterations in p53-dependent apoptosis are critically involved in Akt-mediated chemoresistance (6,14,18). Thus, the role of Akt serine/threonine kinase and its anti-apoptotic substrate XIAP (6,16) in bladder cancer cells undergoing apoptosis after cisplatin treatment has been investigated.

Figure 2. Activation of apoptosis after cisplatin administration, for 24 h, in RT4 and T24 human bladder cancer cells, as evinced by Western blotting. The drug induced a higher dose-dependent cleavage of Caspase-8 (also processed in the RT4 cells treated with the lower drug concentration), Caspase-9 and Caspase-3 family members, and the Caspase repertoire representative substrates PARP and Lamin A/C, as well, in both cell types. However, by comparing the C/P respective ratios for all the examined proteins, a significant relative resistance of T24 cells to cisplatin-mediated apoptosis was unambiguously recognized. Human β-Actin was used as a protein of reference.

Figure 3. Cisplatin-induced accumulation and serine phosphorylation of wild-type p53 protein (left arrows), but not its mutant counterpart (right arrows), in human bladder cancer cells, as demonstrated by Western blotting. Both drug doses (1 and 10 μg/ml) were able to strongly activate the mechanisms controlling p53 protein levels and phosphorylation events at Ser15 and Ser392 exclusively in RT4 (wild-type p53), but not in T24 (mutant p53) cells. Brackets denote the cleaved forms of the wild-type p53 protein.
treatment (Fig. 2) was examined through a Western blotting approach (Fig. 4). In contrast to RT4 cells, T24 were characterized by strongly upregulated levels of phosphorylated Akt on a serine residue at position 473 (Ser473), which could be tightly associated with the different malignancy grade of the two cell lines. The RT4-specific absence of a constitutively activated Akt form, combined together with the generation of two low molecular weight (35 and 40 kDa, approximately) Akt cleaved protein fragments in RT4, but not in T24 cells, after 10 $\mu$g/ml cisplatin administration, presumably operating as dominant negative or ‘squelching’ isoforms, could justify the enhanced chemoresistance of T24 to cisplatin cytotoxic activity (Figs. 1, 2 and 4).

Consistent with the observed chemosensitivity profile, the XIAP anti-apoptotic mediator was shown to be cleaved into low molecular weight fragments, with a prominent moiety of 20 kDa, specifically in RT4 cells treated with 10 $\mu$g/ml cisplatin. The ~90 kDa protein band, exclusively detected in these cell lysates, likely represents a ubiquitinated form of XIAP, thus indicating the grade-specific activation of a general ubiquitination mechanism targeting proteins to proteasome for degradation (16,27). The highest molecular weight band could comprise a XIAP-like homologue, whose functional importance remains to be determined. However, in T24 cells, a drug dose-dependent reduction of XIAP expression levels was observed, mainly characterized by a complete absence of protein fragmentation and ubiquitination signs (Fig. 4).

Cisplatin-induced cleavage of Hsp90 by Granzyme B. Since Hsp90 critically regulates the conformational maturation and stability of p53 and Akt proteins, and has recently emerged as a critical target for cancer therapy (28,29), we attempted to examine its role in cisplatin-mediated apoptosis of bladder cancer cells through a Western blotting approach. As shown in Fig. 5, 10 $\mu$g/ml of cisplatin administration resulted in RT4-specific generation of a major Hsp90 cleaved form (a few minor ones could be also observed) with an approximate molecular weight of 65 kDa. This truncated protein product could presumably function in a dominant-negative manner against native Hsp90, thus inducing structural amputations of critical apoptosis mediators (i.e., p53, Akt and XIAP; Figs. 3 and 4), deregulating their functions and/or targeting them to proteasome for degradation (28).

It has been recently reported that Hsp90 represents a bona fide substrate of Granzyme B in vitro (30). Interestingly, the strong induction of Granzyme B protein levels after treatment of RT4 cells with 10 $\mu$g/ml cisplatin strongly suggests its essential in vivo role in the drug-mediated proteolytic cleavage of Hsp90. In contrast, T24 cells were characterized by a drug dose-dependent Granzyme B down-regulation, clearly justifying their resistance to cisplatin-induced Hsp90 proteolytic processing (Fig. 5).

Cisplatin-induced transcriptional responses implicated in mitochondria-mediated and membrane death receptor-emanated apoptotic pathways. Among the majority of genes examined through a semi-quantitative RT-PCR approach, only the ones depicted in Table I displayed prominent alterations in transcriptional activity. Based on their gene expression profiles after drug administration, three major categories can be recognized: a) genes regulating apoptosis in p53-independent...
manner (Fig. 6); b) p53-dependent genes involved in apoptosis (Fig. 7); and c) genes controlling cisplatin uptake and efflux regardless of p53 genetic status (Fig. 8).

As shown in Fig. 6, and using GAPDH as gene of reference, both Bik and Bim genes were notably upregulated in RT4 and T24 cells treated with 10 μg/ml cisplatin. On the contrary, Bcl-2 expression levels were severely attenuated in the two cell lines examined, only in the higher drug dose, whereas Bcl-XL and Mcl-1 gene activities remained unaffected under both cisplatin concentration conditions (data not shown). Given the pro-apoptotic role of Bik and Bim proteins and the anti-apoptotic activity of Bcl-2 gene product, all critically regulating mitochondria physiology (26,31), the obtained expression profiles strongly suggest a scenario of drug-induced apoptosis through perturbation of mitochondria function. The p53-independent group of apoptotic genes also includes the FAP-1 (PTPL1) gene, coding for an inhibitor of Fas-mediated apoptosis (32,33), which was significantly suppressed in grade-independent manner upon 10 μg/ml of cisplatin exposure (Fig. 6).

However, the membrane death receptor pathways seem to follow grade-specific activation profiles. After 10 μg/ml cisplatin administration, the pro-apoptotic genes Fas (APO-1/CD95), Fasl (CD95L), TRAIL and Caspase-10 were notably upregulated in RT4 cells, while in T24 they remained almost unaffected (albeit a slight downregulation could be observed for Fas and Caspase-10), exempting Fasl and TRAIL.

As shown in Fig. 5, and using GAPDH as gene of reference, the higher drug dose was able to significantly induce the Granzyme B protein expression levels and the generation of a major ~65 kDa Hsp90 cleaved fragment (the thick band at the bottom of the bracket) exclusively in RT4 (grade I) but not in T24 (grade III) cisplatin-treated cells.
significant drug-mediated reduction of FasL expression and the severe attenuation of TRAIL transcriptional activity in T24 cells indicate (together with Fas and Caspase-10 expression profiles) a high grade-specific functional impairment of distinct membrane death receptor systems regulating apoptosis (Fig. 7). Fas, Fasl, Puma and Caspase-10 seem to be activated in p53-dependent fashion, in accordance with previous reports (34-37), whereas TRAIL must follow different, and likely p53-independent (38,39), mechanisms controlling gene activity. Moreover, the RT4-specific induction of Puma after drug exposure strongly suggests an essential role of p53 target genes regulating mitochondria functions in cancer chemoresistance (Fig. 7) (26,34).

p53-independent transcriptional activities associated with cisplatin trafficking. A major determinant of resistance to cisplatin is the diminished accumulation of the drug in a cell. Tolerance to cisplatin can be acquired by critically modulating, among others, the drug uptake and efflux cellular processes (7,8,40,41). As shown in Fig. 8, the ATP7A, ATP7B and MRP1 (ABCC1) genes, encoding for membrane transporters decisively implicated in drug efflux (7,8,42-46), were significantly downregulated in both cell types after 10 μg/ml

Figure 8. Examination of gene activity implicated in cisplatin trafficking after drug administration, for 24 h, in human bladder cancer cells (RT4 and T24), as evinced by semi-quantitative RT-PCR. In contrast to CTR1 function (critically involved in cisplatin uptake) that remained unaffected, the genes associated with drug efflux, ATP7A, ATP7B and MRP1, were notably downregulated in response to the higher dose of cisplatin, exhibiting p53-independent expression profiles. The gene names are shown on the left and their respective RT-PCR product sizes on the right.

Figure 9. Schematic representation of an integrated molecular interaction map concisely depicting the herein examined regulators critically implicated in cisplatin-mediated apoptosis of RT4 and T24 human bladder cancer cells. Exposure to cisplatin results in the activation or repression of distinct p53-dependent and p53-independent mechanisms tightly related to induction of apoptosis and drug resistance. Proteins are denoted by bold-type fonts, whereas genes are represented by non-bold- and italic-type letters.
cisplatin administration. In contrast, the expression levels of CTR1 (SLC31A1) gene, whose cognate protein regulates the import of cisplatin into a cell (7,8,42,44,46), remained unaffected after drug exposure of both RT4 and T24 cells (Fig. 8). Therefore, cisplatin-induced toxicity in bladder cancer cells could be critically associated with p53-independent attenuation of certain drug efflux systems, eventually resulting in increased intracellular concentration of cisplatin.

Discussion

A major holdback to successful cancer chemotherapy is the, frequently observed, development of acquired resistance to drug exposure, which, thereby, renders tumor cell populations refractory to the applied regimenS, eventually resulting to relapse of the disease. Cellular resistance to cisplatin cytotoxic activity seems to be a multifactorial process, mainly implemented through two broad types of mechanisms: a) prevention of the adequate amounts of drug from reaching and binding to the target DNA molecule, due to decreased cisplatin uptake, increased cisplatin efflux, upregulated detoxification and augmented DNA repair, and b) failure of cell death and increased survival, despite the drug-induced DNA damage, through severe deregulation of the apoptosis-related signaling pathways (6-11,26,47,48).

Apoptosis is the prevailing response of cells to chemotherapeutic agents. Two major apoptotic pathways have been described for mammalian cells. The first one involves Caspase-8, which is activated through membrane death receptor-mediated extrinsic pathways, whereas the second one implicates mitochondria-emanated intrinsic pathways, being mainly characterized by activation of Caspase-9 via Cytochrome c release into the cytosol and apoptosome formation (an Apaf-1- and ATP-dependent process). Subsequently, activated Caspase-3, as the major effector component of both pathways, initiates a program of proteolytic cleavages of critical cellular proteins, including PARP and Lamin A/C, eventually resulting to apoptosis (6,26,33,47,49).

Indeed, here, we showed that exposure of human bladder cancer cells to cisplatin resulted in the generation of cleaved Caspase-8, Caspase-9, Caspase-3, PARP and Lamin A/C proteins, thus undoubtedly demonstrating the activation of apoptosis (Fig. 2). Both the membrane death receptor- and mitochondria-dependent pathways seemed to be engaged in the RT4 and T24 cell types, albeit in a grade-specific manner. Interestingly, through the comparison of their respective C/P ratios, T24 proved to be significantly more resistant to cisplatin administration than RT4 cells, strongly corroborating their MTT assay-based differential cytotoxic responses (Fig. 1). Moreover, in contrast to the activation of Caspase-8 even in the lower drug dose of RT4-treated cells, which unambiguously confirms their increased sensitivity to the drug, T24 were not only unable for a respective response, but they were also missing the 18 kDa small fragment, likely implying an inability to form the mature and fully active tetramer of Caspase-8, and an attenuation of the Caspase-8-mediated downstream signaling pathways. In agreement with our results, proteolytic processing and activation of initiator and effector Caspases have been also reported before in Jurkat T leukemia cells, MCF-7 breast cancer cells and human osteosarcoma (HOS) cells after cisplatin administration (6,49-51). Notably, cisplatin apoptotic activity in Jurkat T leukemia cells was shown to be independent of membrane death receptors, while mitochondria contribution was definitely required (49). However, depending on the cellular model system used, cisplatin-induced apoptosis could also involve certain Caspase-independent pathways, as it has been previously described for ovarian cancer cells and renal proximal tubular cells (RPTC) (6,52,53). In toto, we reveal that clinically relevant doses of cisplatin can induce apoptosis in bladder cancer cells through the activation of Caspase repertoire, whereas relative tolerance to the drug can arise from downregulation of membrane death receptor-emanated apoptotic pathways.

p53 is a fundamental tumor suppressor protein that plays a crucial role in a variety of biological processes including cell-cycle arrest and induction of apoptosis in response to cellular stress (6,54). Sensitivity to cisplatin has been tightly associated with the presence of wild-type p53 activity in a National Cancer Institute (NCI) collection of sixty human cancer cell lines (6,55). Furthermore, tumor cells lacking functional p53 were more resistant to cisplatin than cells containing wild-type p53, but could be sensitized upon reconstitution with the proficient p53 protein (6,56-58). Nevertheless, in certain cellular systems no or even negative correlation between response to cisplatin and p53 status has been previously observed. For example, in SaOS-2 osteosarcoma cells p53 activity was adequately tied in with increased resistance to cisplatin under low serum conditions, whereas the absence of p53 in testicular teratocarcinoma cells did not alter cellular sensitivity to the drug (6,59,60).

We, herein, reveal that the grade-specific cytotoxic and apoptotic responses of RT4 and T24 human bladder cancer cells to cisplatin are tightly associated with the drug-induced functional activities of p53 protein (Fig. 3). It seems that the missing critical tyrosine 126 (Y126) dramatically affects the molecular physiology of the p53 mutant protein, thus prohibiting its competence to be phosphorylated at Ser15 and Ser392 target residues upon cisplatin exposure of T24 cells. Interestingly, certain p53 natural mutants from human tumors, harboring changes elsewhere from the decisive serine positions, such as [Ile203]p53 and [Ala143]p53, exhibit altered Ser15 and Ser392 phosphorylation profiles (61). In RT4 cells, the cisplatin-induced phosphorylation at Ser15 results in the structural stabilization and intracellular accumulation of p53 protein, by likely disrupting its interaction with the MDM2 negative regulator, which otherwise targets p53 for ubiquitin-mediated degradation. This stress signal-dependent increase in p53 half-life time from minutes to hours promotes distinct transcriptional programs tightly regulating apoptosis (13,20,27,34,62,63). Alternatively, Ser15 phosphorylation could be implicated in promoting p53 interaction with B56b-PP2A phosphatase, thereby leading to Thr55 dephosphorylation, and subsequent induction of p53 protein stabilization, as recently described (64). On the other hand, the cisplatin-mediated phosphorylation of the wild-type p53 at Ser392, exclusively observed in RT4 cells, should strongly potentiate its DNA binding activity through a putative stabilization mechanism of the p53 tetramer form (13,65). Since MDM2 constitutes a bona fide p53 target gene (13,20,34), the
progressive accumulation of functional p53 upon cisplatin exposure could result in overproduction of MDM2 protein that acts as a ubiquitin ligase to effectively degrade p53 through proteasome targeting (20,27,63), thus likely explaining the cleaved p53 forms detected in the higher drug dose (Fig. 3).

Although it remains to be determined, in RT4 cells, one (or more) of the ATM, ATR and DNA-PK kinases could be likely implicated in the cisplatin-induced phosphorylation of p53 at Ser15, whereas Ser392 phosphorylation might be implemented by one (or more) of the p38 MAPK, CDK9 and CK2 kinases (65-67). Accordingly, a role of ATR/Chk2 signaling in p53 activation and subsequent apoptosis has been recently demonstrated in cisplatin-treated renal cells, thereby further illuminating the drug-induced nephrotoxicity mechanisms (15). ATR contains a putative Akt phosphorylation site (RRRLSS), and as such its function could be strongly regulated by the activated Akt kinase (18). Indeed, in ovarian cancer cells, inhibition of functional Akt potentiated total and phosphorylated p53 contents upon cisplatin exposure, whereas its activation suppressed drug-mediated phosphorylation of p53 (18). Thus, an (indirect) inhibitory role of the activated Akt kinase in p53 [especially when mutated (i.e., in T24 cells)] phosphorylation status could be likely envisioned for bladder cancer cells undergoing cisplatin treatment. In the same study, it was also shown that cisplatin induced phosphorylation of numerous p53 residues in chemosensitive ovarian cancer cells, but not in their respective chemoresistant variants, thereby suggesting that altered p53 phosphorylation profiles may significantly contribute to the acquisition of drug resistance during therapy. Moreover, the p53 phosphorylation status at Ser15 after cisplatin administration has been severely compromised in a chemorefractory clone of the A2780 ovarian cancer cell line (13), whereas ionizing radiation, applied as another typical DNA damaging factor, induces apoptosis in bladder cancer cell lines in p53-dependent manner (22). However, the relative chemosensitivity of RT4 cells to cisplatin could not solely be attributed to the marked phosphorylation of wild-type p53 at Ser15 and Ser392, but additional determinants are required for the implementation of cisplatin-mediated apoptosis in bladder cancer cells. This scenario is strongly supported: a) by the ability of T24 cells to undergo apoptosis, albeit relatively extenuated, after drug treatment, despite the absence of overproduced and functional p53 protein, and, vice versa, b) by the inability of phosphorylated p53 to promote robust apoptosis of RT4 cells under lower cisplatin concentration, as compared to the higher drug dose respective response. Nevertheless, when total and phosphorylated p53 protein together with its respective cleaved forms seem to exceed critical thresholds of intracellular concentrations (i.e., in the higher dose of cisplatin) a reinforced apoptotic process could likely occur (Fig. 3).

Several observations indicate that Akt can protect cells from apoptotic death induced by various stress stimuli, including cisplatin (6,47). Inactivation of Akt by ectopic expression of its negative regulator PTEN in tumor cells enhances drug-induced apoptosis, whereas transfection of constitutively active Akt in lung cancer cells reduces topotecan-mediated apoptosis (47,68). Moreover, fibroblasts overexpressing Akt become chemoresistant to staurosporine and etoposide apoptotic activities (47,69,70). During tumor development, Akt can be activated by a number of mechanisms, including downregulation of PTEN (a dual-specificity phosphatase), overactivation of the PI3K (PI-3 kinase) upstream (positive) signaling mediator, Akt gene amplification and acquisition of somatic mutations in Akt critical residues, such as the glutamic acid to lysine substitution at amino acid position 17 (E17K) that promotes cellular transformation and induces leukemia in mice (47,71). Therefore, the constitutively phosphorylated at Ser473, and presumably active, Akt form could be essentially implicated in the highly malignant behavior of T24 cells and in their partial resistance to cisplatin apoptotic activity. In contrast, the absence of a functional Akt in RT4 cells must be tightly associated with their low grade of tumorigenic potency and enhanced sensitivity to the drug (Fig. 4). Interestingly, it has been previously reported that the Akt phosphorylation content is strongly correlated with the invasiveness of urethelial cell lines (72). In contrast to RT4, the highly invasive T24 cell line harbors a missense point mutation at codon 48 (substitution of Ile for Asn) in the, critical for enzymatic activity, α2 helix of the PTEN dual-specificity phosphatase (73). Thus, the putative reduction of Akt dephosphorylation due to PTEN functional impairment (73), along with the increased PI3K catalytic activity levels, as elsewhere assessed by the specific inhibitor LY294002 (72), could both reliably account for the strong and constitutive expression of the phosphorylated at Ser473, and consequently activated, Akt apoptotic regulator, exclusively in T24 cells (Fig. 4).

Even though the RT4 cells do not express any phosphorylated at Ser473 Akt form, the generation of Akt and XIAP cleaved fragments could critically contribute to the higher drug dose-induced apoptosis. In ovarian cancer cells, XIAP can be phosphorylated at Ser87 by Akt, being, thereby, stabilized through inhibition of both its (XIAP) auto-ubiquitination and cisplatin-mediated ubiquitination activities (6,16). It seems that XIAP degradation is an important mechanism to regulate its intracellular steady-state functional levels and determine sensitivity to cisplatin exposure. The anti-apoptotic activity of XIAP is exerted through direct inhibition of Caspase-3 and Caspase-7, and modulation of the Bax/Cytochrome c pathway by blocking Caspase-9 (12,74). In ovarian cancer cells, XIAP overexpression is associated with reduced cisplatin-stimulated Caspase-3 activity and apoptosis, while downregulation of XIAP is considered as an effective mean to overcome chemoresistance in the same cells that express either endogenous or reconstituted wild-type p53 protein (6,14,16,74,75). It seems that RT4 and T24 cell lines engage distinct mechanisms to regulate XIAP in response to cisplatin. Under the higher drug dose conditions, RT4 are characterized by cleaved XIAP forms (cleavage pattern), whereas in T24 a significant reduction of the unprocessed protein is observed (complete degradation pattern) (Fig. 4). Since a relatively similar grade-specific pattern can be recognized for Akt as well, a putative mechanism of an Akt-dependent phosphorylation of XIAP that is able to prevent cisplatin-induced XIAP cleavage could be rationally adopted for bladder cancer cells (6,16). However, the constitutively active Akt form in T24 cells does not seem to protect XIAP from complete degradation that is mainly defined by
the absence of cleaved fragments, but drug dose-dependent reduction of the unprocessed protein expression levels. Thus, XIAP might play a master regulatory role in cisplatin-induced apoptosis of T24 cells. When XIAP intracellular protein levels drop off a certain threshold, the Caspase repertoire can not be any more efficiently inhibited and apoptosis is immediately activated (74,75). On the other hand, the cleaved XIAP forms detected in drug-treated RT4 cells could not only abolish XIAP anti-apoptotic potential, but could also function as negative (or positive) regulators of several critical components suppressing (or promoting) cell death, including Akt. The RT4-specific XIAP cleaved pattern could be produced by either a ubiquitin-mediated process, likely associated with the higher molecular weight band exclusively detected in 10 μg/ml cisplatin (Fig. 4; one asterisk), or a Caspase-3-dependent mechanism, as previously demonstrated during Fas-stimulated apoptosis (76). Interestingly, it was previously shown that active Caspase-3 was able to cleave Akt in vitro and this effect could be blocked by the cognate Caspase inhibitor (75). According to this model, the Akt cleaved forms could function in a dominant negative manner, thus inhibiting the endogenous Akt activity and consequently implying a role of the non-phosphorylated Akt in suppressing apoptosis of RT4 cells. Alternatively, these small forms might illegitimately interact with distinct apoptotic regulators, such as XIAP protein, through a ‘squelching’ process, sequentially excluding them from the molecular anti-apoptotic network and eventually rendering them susceptible to functional inhibition or degradation. In contrast, the phosphorylation of Akt at Ser473 seems to provide protection against cisplatin-mediated cleavage of both Akt and its target substrate XIAP (6,14,16), therefore offering T24 cells the advantage of relative chemoresistance.

Similarly to ovarian cancer cells (14), the wild-type p53 function in RT4 cells is absolutely required for the cisplatin-induced apoptotic effects of Akt and XIAP structural amputations. However, in drug-treated T24 cells XIAP downregulation could be partly counterbalanced by the non-functional p53 and constitutively active Akt, thereby conferring certain levels of resistance to cisplatin apoptotic activity. Interestingly, in ovarian cancer cells, Akt was able to attenuate the cisplatin-induced mitochondrial p53 accumulation and p53-dependent release of Smac apoptotic protein [a known counteractor of IAPs, including XIAP (26)] into the cytosol, strongly desensitizing cells from drug cytotoxic action (17). Hence, the functional p53 status should be considered as a major determinant of the Akt- and XIAP-mediated chemoresistance frequently acquired during bladder cancer therapy.

Beyond its genotoxic activity, cisplatin critically modulates the expression profile of Granzyme B in response to cisplatin (Figs. 3 and 5). Intriguingly, delivery of Granzyme B to melanoma cells (T1) resulted in the activation of ATM and p38 MAPK stress kinases and subsequent phosphorylation of p53 at Ser15 and Ser37 residues, providing insight into the functional relationship between Granzyme B and p53 during cellular disintegration (81). Therefore, the grade-dependent expression profile of Granzyme B in response to cisplatin administration might be functionally associated with the p53 genetic status of RT4 and T24 cells. As such, when Granzyme B is overexpressed in RT4 cells, it could significantly contribute to p53 protein accumulation and phosphorylation, while its downregulation in T24 cells might hamper p53 (particularly when mutated) responses to cisplatin (Figs. 3 and 5). Furthermore, it seems that the cisplatin-mediated decrease of Granzyme B in T24 cells allows the protection of Hsp90 from proteolytic cleavage, thereby retaining the structural integrity of certain anti-apoptotic clients (i.e., Akt) under cisplatin exposure conditions, and eventually providing some degree of cellular resistance against them. Whether Granzyme B represents a bona fide target gene of the activated p53 protein is an interesting speculation that needs to be further explored (34).

Part of the DNA damage response can be coordinated, besides p53, by additional pathways of apoptosis guided
by the E2F1 transcription factor (82,83). After cisplatin exposure, E2F1 protein can be stabilized in the drug-treated cells by distinct mechanisms, including ATM-mediated serine phosphorylation, increased CDK2 kinase activity and, likely, P/CAF-induced lysine acetylation (83-86). Interestingly, the accumulation of E2F1 protein in mouse embryo fibroblasts (MEFs) following cisplatin treatment seems to occur independently of their p53 genetic status (84,86). E2F1 activation results in the transcriptional upregulation of critical pro-apoptotic genes, such as Bik and Bim, and the transcriptional repression of distinct anti-apoptotic family members, including Bel-2 (82,83,87-91). According to this, and since the exposed to the higher dose of cisplatin RT4 and T24 cells were both characterized by a Bik and Bim strong transactivation response, paralleled with a severe downregulation of Bel-2 gene (Fig. 6), E2F1 might be critically involved in the cisplatin-mediated apoptosis of bladder cancer cells and, likely, in a p53-independent manner. It seems that both grade I (RT4) and grade III (T24) cell types could undergo cisplatin-induced apoptosis through an E2F1-mediated functional perturbation of the intracellular balance between certain pro-apoptotic (i.e., Bik and Bim) and anti-apoptotic (i.e., Bel-2) components (83). As a consequence, mitochondrial permeability should be increased, allowing the leakage of, among others, the pro-apoptotic molecule Cytochrome c into the cytosol, whereat it could induce the apoptosome formation, maturation of Caspase repertoire and eventually activation of apoptosis (12,26,31,47). Thereby, for cisplatin to operate as an apoptotic inducer in bladder cancer cells, the intrinsic mitochondrial pathway seems to be absolutely required and, moreover, its functional potency must be partly regulated in a p53-independent manner. Interestingly, Bel-2 was found to be frequently overexpressed in a variety of human cancers (12,47,80), while the acquisition of resistance to cisplatin in a T24-derived subclone (T24R2) was previously associated with a strong upregulation of Bel-2 protein levels (92). Moreover, in human lung cancer H460 cells exposed to cisplatin, Bel-2 protein was shown to be downregulated through a dephosphorylation and ubiquitination process, targeting its degradation by proteasome (27,93).

Contrary to E2F1, the p53-dependent transactivation functions can be functionally coupled to the differential sensitivity of RT4 and T24 cells to cisplatin exposure. Distinct p53 target genes, such as Fas, Fasl, Puma and Caspase-10, (15,34-37,94) were transcriptionally upregulated after drug treatment in the p53-proficient RT4 cells, but not in the p53-deficient T24 cells (Fig. 7), whereas both Bax and Noxa (other p53 targets) gene activities in response to the drug remained unaffected (data not shown), thereby allowing the association of T24 relative resistance to cisplatin toxicity with certain p53 target gene product cellular activities. It seems that the presence of a functional wild-type p53 is tightly linked to the efficient induction of the Fas/FasL-mediated apoptosis in bladder cancer cells after cisplatin administration. Indeed, Fas and Fasl cell surface expression can be strongly upregulated after exposure of several cancer cell lines carrying wild-type p53 to cisplatin (33,95), whereas mutant p53 can function as a potent repressor of Fas gene activity (95,96), thus providing p53-deficient or p53-null cells with the advantage of chemoresistance. As previously suggested, the chemosensitivity of solid tumor cells depends on intact apoptotic pathways, involving engagement of the Fas/FasL system and processing of the cognate Caspases (6,97). Thereafter, the relative resistance of T24 cells to cisplatin activity could evolve through a p53-dependent functional attenuation of the Fas/FasL signaling pathway, a scenario that is strongly supported by the absence of a mature and fully active Caspase-8 (since having missed the small cleaved fragment) in T24 cells treated with the higher drug dose (Fig. 2). Interestingly, loss of the Caspase-8 activation pathway has been previously implicated in resistance to cisplatin-mediated apoptosis of HEP-2 cells (human laryngeal cancer) (98). The diminished role of Fas/Fasl system in cisplatin-induced apoptosis has also been suggested before for T24 (92) and Jurkat T (49) cell lines, while null for FADD or Caspase-8 embryonic fibroblasts demonstrated only a partial role of the membrane death receptor pathway in response to chemotherapeutic agents (47).

Furthermore, the absence of TRAIL gene expression and the relative unresponsiveness of Caspase-10 gene (albeit weakly debilitated) to cisplatin exposure (Fig. 7) indicate that, together with Fas/Fasl, the other membrane death receptor system of TRAIL/DR4/DR5/Caspase-8/Caspase-10 should be also functionally attenuated, thus significantly contributing to the T24-specific partial resistance to cisplatin apoptotic activity. Accordingly, T24 cells, when compared to RT4, can exhibit significant levels of resistance to TRAIL-induced apoptosis, and, interestingly, in an Akt-dependent manner (99). Even though both DR4 and DR5 genes [whose cognate proteins operate as TRAIL membrane receptors (12)] follow prominent p53-dependent expression profiles upon cisplatin exposure (data not shown), the grade-specific repression of TRAIL gene activity (Fig. 7) ensures the complete hindrance of TRAIL/DR4/DR5 functional engagement in drug-induced apoptosis of T24 cells. TRAIL gene silencing in T24 cells could be mediated through distinct mechanisms, including grade-dependent transcriptional suppression, DNA methylation and histone deacetylation. Indeed, TRAIL is classified among the typical FOXO family member target genes (39), whose transcriptional activities are negatively regulated by, among others, an Akt-mediated phosphorylation mechanism (100-102). Therefore, the constitutively active Akt form, exclusively observed in T24 cells (Fig. 4), could specifically phosphorylate distinct FOXO transcription factors, rendering them inactive to downstream upregulate their target gene TRAIL. Nevertheless, TRAIL gene activity can be restored in certain cellular cancer model systems by either 5-Aza-2'-deoxycytidine or HDAC (histone deacetylase) inhibitors, clearly demonstrating the critical role of DNA demethylation and/or histone acetylation, respectively, in apoptotic gene regulation (12,103,104). Our findings likely open a new therapeutic avenue to introducing highly efficient apoptosis in p53-mutated malignant cancers, such as bladder cancer, by targeted overexpression and constitutive activation of the Fas, Fasl, and TRAIL apoptotic regulators, only in the tumor area (37).

The p53-dependent expression profile of Puma gene (Fig. 7) strongly supports the implication of its cognate protein product in the acquisition process of RT4 and T24 differential resistance to cisplatin activity. Puma, a bona fide
The p53 target gene (34,35), has been previously associated with cisplatin-mediated nephrotoxicity and renal tubular cell apoptosis through an activated p53-specific mechanism (15, 36). Thereby, in contrast to T24, the putative upregulation and mitochondrial accumulation of Puma, BH3-only, protein in cisplatin-treated RT4 cells could result in a severe neutralization of the Bcl-2 family-based anti-apoptotic functions and a Bax-mediated release of Cytochrome c into the cytosol, followed by Caspase maturation and activation of apoptosis, as previously demonstrated for other cellular systems (15,26,31). Conclusively, a Puma-induced oversensitization of the intrinsic mitochondrial pathway might also critically contribute to the reduced resistance of RT4 cells to cisplatin toxicity.

The group of genes presenting a p53-independent downregulation of the respective transcriptional activities in response to cisplatin contains, besides Bcl-2, the FAP-1, ATP7A, ATP7B and MRP1 distinct members. FAP-1 expression levels were significantly attenuated in both RT4 and T24 cell lines upon exposure to the higher drug dose (Fig. 6), presumably implying a potential role of FAP-1 protein in cisplatin-induced bladder cancer cell apoptosis. However, this could more likely apply to RT4 and not to T24 cells, since their differential Fas/FasL activation dynamics critically determines the functional importance of the cognate downstream mediators in apoptotic signaling. FAP-1 overexpression has been reported to inhibit Fas-mediated apoptosis in certain human malignant cells, by acting as a negative switch in the Fas/FasL pathway, thereby providing them with a marked survival advantage (32,33). Moreover, silencing of FAP-1 gene expression or inhibition of FAP-1 protein tyrosine phosphatase (PTP) activity could abolish tumor cell resistance to Fas-induced apoptosis in a head and neck cancer model system (SCCHN cells) (105). In contrast, FADD protein, another critical component that positively regulates the Fas/FasL apoptotic activity could abolish tumor cell resistance to Fas-induced apoptosis in a head and neck cancer model system (SCCHN cells) (105). In contrast, FADD protein, another critical component that positively regulates the Fas/FasL apoptotic activity could abolish tumor cell resistance to Fas-induced apoptosis in a head and neck cancer model system (SCCHN cells) (105). In contrast, FADD protein, another critical component that positively regulates the Fas/FasL apoptotic activity could abolish tumor cell resistance to Fas-induced apoptosis in a head and neck cancer model system (SCCHN cells) (105). 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in vivo biomarkers in order to develop a molecular platform that could reliably monitor the responses of human bladder cancer cell populations to cisplatin therapeutic actions, thereby providing clinical protocols with novel and powerful genetic tools for a more efficient and successful management of human urothelial malignancies.

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