

Molecular mechanisms of cisplatin resistance in bladder cancer

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Metastatic disease is the most common mechanism of death in patients with advanced bladder cancer. As for most solid tumors, chemotherapy remains the only realistic option for palliating or curing metastatic disease. However, bladder cancer is characterized by chemoresistance. Only modest response rates are obtained using multiagent regimens including cisplatin. These low response rates and the toxicity of these regimens limit their use to patients at highest risk. Here, we review the molecular mechanisms of cisplatin resistance. These include methods to reduce cisplatin bioavailability within a cell, and defects in the machinery that produces cell death following cisplatin-induced DNA damage. While overcoming these mechanisms is a potential therapeutic approach that can increase response rates, in the short term this knowledge could be used to predict response in individual tumors.

KEYWORDS: apoptosis • bladder cancer • cisplatin • cisplatin resistance • DNA repair • drug resistance • epigenetics

Bladder cancer is the fifth most common malignancy in the USA, with 70,530 new cases and 14,680 deaths in 2010 [1]. The relapsing nature of this cancer makes it one of the most expensive human malignancies to treat [2]. Estimates suggest that the disease costs approximately US\$3.7 billion/per annum in the USA, and the majority of this figure is spent on diagnosis and surveillance [3]. Most tumors are histologically typed as urothelial cell carcinoma (UCC) and these are best separated into those with low- and high-grade cellular differentiation. These distinct tumor phenotypes have different molecular pathways, different clinical behavior and therefore require different clinical management [4–6]. Low-grade cancers are primarily managed by bladder-sparing techniques including endoscopic resection and intravesical chemotherapy. High-grade cancers require close monitoring while confined to the urothelium, and radical treatment if resistant to therapy or once they develop muscle invasion [7]. While low-grade cancers are the most common (70%) and rarely progress beyond the urothelium, patients with high-grade tumors have a poor prognosis and once muscle invasion has occurred the overall survival at 5 years is approximately 50% [8,9]. For locally advanced or metastatic disease the outlook is worse, with only 10–15% of patients surviving 5 years [10]. Improvements in survival

are achieved through radical treatment for high-risk non-muscle invasive disease and with systemic chemotherapy [11]. UCC is characterized by chemoresistance and the best response is seen using multidrug platinum-based regimens. Adjuvant chemotherapy results in a 25% relative reduction in the risk of death [12], while preoperative treatment produces a 13% relative reduction in the risk of death, leading to an absolute 5% increase in 5-year survival [13,14]. Drug toxicity and low response rates mean that chemotherapy is restricted to the fittest patients and those with the highest relapse risk. Here, we describe the known molecular mechanisms for cisplatin resistance in UCC. Many of these are shared among solid cancers and this knowledge is vital if mechanisms to bypass drug resistance are to be found. The cellular events which underpin cisplatin resistance in cancer are multiple and affect numerous cellular pathways (for a review of mechanisms of cisplatin resistance in all tumor types see [15]).

Cisplatin & bladder cancer

Since its discovery by researchers studying the effect of electric fields on *Escherichia coli* growth [16], cisplatin (*cis*-diamminedichloroplatinum [II]) has been adopted as a chemotherapeutic agent against many types of metastatic tumors. In particular, cisplatin is the key

component of chemotherapeutic regimes used to treat UCC, either as part of the methotrexate, vinblastine, adriamycin, cisplatin (MVAC) or gemcitabine and cisplatin (Gem-Cis) drug combinations. The latter is currently the most popular as it has similar efficacy to MVAC, but with lower toxicity.

While cisplatin can react with DNA, RNA and protein, it appears that DNA is its primary cellular target [17]. Upon entering a cell and undergoing activation by the replacement of its two chloride ligands with water molecules, cisplatin is able to react with the N7 atoms of purine bases in DNA to form a variety of adducts including inter- or intra-strand crosslinks (where a single cisplatin molecule is bound to the N7 atom of two purine bases on the same strand or opposing strands respectively), and monofunctional adducts (where cisplatin binds to a single purine base) [18]. The final cellular outcome of the cisplatin-mediated DNA damage is cell death via the activation of the apoptotic pathway [19].

Cell culture models of cisplatin resistance in UCC

Resistance to cisplatin represents a serious obstacle to the treatment of UCC, and to better understand this resistance many cell culture models of cisplatin resistance have been generated. Typically, UCC cells are treated with increasing concentrations of cisplatin until they become tolerant to relatively high concentrations of cisplatin (generally approximately 12 μ M). The resulting resistant cells display similar doubling times to their parental cells and often display cross-resistance to carboplatin and methotrexate, but not doxorubicin [20]. Resistant cells undergo distinct morphological changes, such as an increased number of double membrane and pinocytic vesicles, compared with their parental lines [21]. Reports of laboratory-generated cisplatin-resistant cell lines have also described resistance to the alkylating agent temazolamide [22]. Cisplatin resistance has also been acquired in culture by UCC cells treated for long periods with mitomycin C [23], although this phenomenon was not observed in cells cultured for long periods with BMY25067, a mitomycin C analog [24]. The presence of bladder cancer stem cells, or tumor-initiating cells (TICs) has also been investigated in bladder cancer cell lines. A recent study established that a small subset of cells in a population of the bladder cancer cell lines T24, 5637 and JTC30 characterized by increased cell surface CD44 constituted TICs. These cells displayed properties typical of TIC, including increased tumorigenic capacity and increased resistance to cisplatin [25], suggesting a mechanism whereby a cisplatin-resistant subpopulation of TICs survive the initial chemotherapy and then facilitate the recurrence of the disease.

Reduced intracellular availability: uptake, efflux & sequestration of cisplatin

Cisplatin-resistant cells often have reduced intracellular platinum accumulation following drug exposure. This may result from reduced uptake, increased drug export or intracellular sequestration (FIGURE 1). While cisplatin uptake is mediated through the copper transporter protein Ctr1 [26], efflux is performed by two other copper transporting p-type adenosine triphosphatases (ATP7A [27]

and ATP7B [28]). Not surprisingly, changes in the expression of these proteins have been implicated in cisplatin resistance and poor patient survival in some types of cancer, most notably ovarian cancer [29]. There are currently no data to support their role in cisplatin resistance in UCC.

P-glycoprotein/MDR1

The ATP-binding cassette protein P-glycoprotein (pGP) is a product of the *MDR1* gene and is frequently associated with multidrug resistance in human cancer. Cisplatin is not a substrate for pGP [30], and thus many studies of pGP expression in UCC have failed to show an association with response to cisplatin-based chemotherapy [31–33]. While a recent large study in UCC has suggested increased pGP expression is associated with a poor response to MVAC [34], this effect is probably due to pGP-mediated efflux of coadministered drugs within this regimen, rather than cisplatin (doxorubicin [35], vinblastine [36], and in certain circumstances methotrexate [37], are all well known substrates of pGP). Indeed, tumors from patients treated with MVAC have been found to express higher levels of pGP compared with untreated patients [38,39]. *In vitro* studies that have generated high-pGP expressing doxorubicin-resistant UCC cells showed no increase in cisplatin resistance [40,41].

Multidrug resistance protein

Multidrug resistance protein (MRP) does not seem to have an effect on the response of UCC to cisplatin. A study by Clifford *et al.* on 25 patients with this cancer showed that MRP mRNA levels decreased as tumor grade and cisplatin resistance increased [42]. They also showed that MRP mRNA levels remained unchanged immediately following cisplatin treatment.

Canalicular multispecific organic anion transporter/MRP2

The canalicular multispecific organic anion transporter (cMOAT)/MRP2 pump may also play a role in the modulation of cisplatin resistance in UCC. In the initial paper describing its discovery [43], cMOAT was shown to be upregulated in a variety of cisplatin-resistant derivatives of human cancer cell lines, including T24 UCC cell lines, where its expression correlated with decreased cisplatin accumulation. Further studies have since identified increased cMOAT/MRP2 expression in a wide variety of other cisplatin-resistant cell culture models [44,45]. As with pGP, bladder tumor material from patients treated with MVAC displayed increased MDR2 expression compared with untreated patients [39]. Work further implicating increased cMOAT/MRP2 expression in cisplatin-resistant cells has not involved UCC cell lines, but reducing cMOAT/MRP2 expression using ribozymes has been shown to restore cisplatin sensitivity in cisplatin resistant adrenocortical carcinoma and melanoma cell lines [45]. This suggests increased cMOAT/MRP2 expression in UCC may contribute to cisplatin resistance.

MRP2 requires glutathione as a cofactor, and its role in protection from the cytotoxic effects of cisplatin may be a result of its ability to transport glutathione/cisplatin conjugates across the cell membrane (see below) [46].

Detoxification by intracellular thiols

While the N7 atom of purine bases in DNA constitute the main therapeutic target for cisplatin, the vast majority of cisplatin molecules will react with other ligands before ever reaching the nucleus [47]. Cisplatin has a weaker affinity to nitrogen donors than sulfur donors [48]. As such, intracellular thiols such as glutathione and metallothioneins represent major targets for interacting with cisplatin and mediation of the amount of cisplatin that reaches the nucleus.

Metallothioneins are a class of low-molecular weight, cysteine-rich proteins that are known to detoxify heavy metal ions and protect cells against oxidative stress. While mainly expressed in the liver, kidney and intestine, metallothionein is known to be expressed in UCCs [49], and deliberate exposure of mice bearing UCC xenografts to zinc has been shown to cause an associated reduction in cisplatin sensitivity alongside the expected increase in metallothionein expression [50]. UCC mouse xenografts have also been sensitized to cisplatin following treatment with propargylglycine, an inhibitor of metallothionein synthesis [51].

Reports have shown that bladder tumors with low or undetectable levels of metallothionein expression were more likely to respond completely to cisplatin-based chemotherapy [32,52]. A subsequent, larger study has reinforced metallothionein as a significant predictor of the response of metastatic UCC to cisplatin-based chemotherapy [33].

Glutathione

Glutathione (GSH) is a thiol containing tripeptide (Glu-Cys-Gly), present at concentrations of approximately 1–8 mM in mammalian cells [53]. It is synthesized from constituent amino acids in a two-step pathway, catalyzed by γ -glutamylcystine synthetase and GSH synthetase sequentially. Cisplatin has been shown to form conjugates with GSH [54], a process which can be catalyzed by the action of glutathione-S-transferases (GSTs) such as GSTP1-1 [55]. Increased expression of enzymes concerned with GSH synthesis and conjugation are implicated in the generation of cisplatin resistance. In addition to this, GSH can work in concert with cMOAT/MRP2 to pump GSH–cisplatin conjugates out of cells in an ATP-dependent manner [56]. Resistance to cisplatin through this mechanism is entirely GSH-dependent [46]. Conversely, in some cell types, most notably SR3A small-cell lung cancer cells, increased GSH levels have been shown to sensitize cells to cisplatin treatment through increased expression of the Ctr1 protein, which is responsible for cisplatin import [57].

Increased levels of intracellular glutathione are frequently observed in cisplatin-resistant tumors and resistant bladder cell

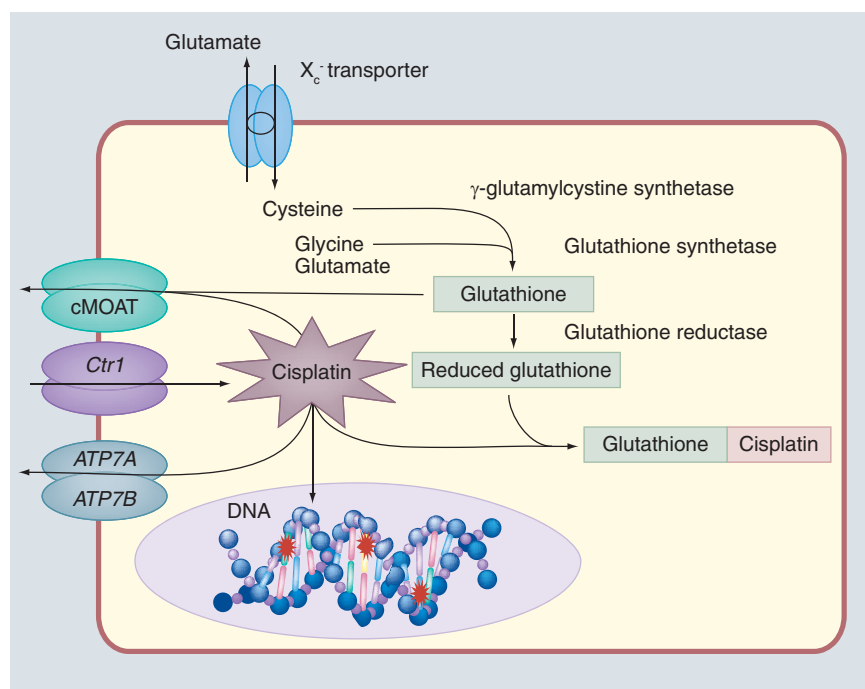


Figure 1. Cisplatin resistance may be mediated by reduced intracellular availability. This can be achieved by reducing cisplatin import (silencing of *Ctr1*), increased efflux (through *ATP7A*, *ATP7B* or *cMOAT*) or intracellular sequestration with a thiol (such as glutathione or thioredoxin).

lines [58,59]. The expression of GST is unchanged in sensitive and resistant sub-lines, suggesting another cause for the increase in intracellular glutathione [60]. Screens of UCC cell lines have shown that higher levels of glutathione and glutathione-related enzymes strongly correlate with cisplatin resistance, and that glutathione depletion with the glutathione neosynthesis inhibitor buthionine sulfoximine reduces the IC_{50} of cisplatin in these cells [61].

Masters *et al.* found upregulation of GSH and metallothionein in the RT112 UCC cell line following the development of cisplatin resistance [62]. When compared, GSH levels were found to be lower in testicular germ cell tumor cells than in UCC cell lines. Testicular tumors generally respond well to cisplatin treatment.

Studies on J82/MMC, a UCC cell line resistant to mitomycin C, showed that while mitomycin C resistance was primarily mediated by reduced cytochrome p450 expression [63], there was also an associated increase in cisplatin resistance. This was independent of platinum accumulation, which was attributed to increased levels of cellular thiols mediated by increased π -type GST activity and an increased expression of metallothionein IIa mRNA [24].

Thioredoxin has also been shown to play a role in the cisplatin resistance of UCC cells. Levels of thioredoxin mRNA and protein were markedly higher in cisplatin-resistant derivatives of RT112 and KK47 UCC cell lines (and the PC3 prostate cancer cell line) [64], and where thioredoxin expression was reduced (using the expression of antisense thioredoxin) an increase in cisplatin sensitivity was observed.

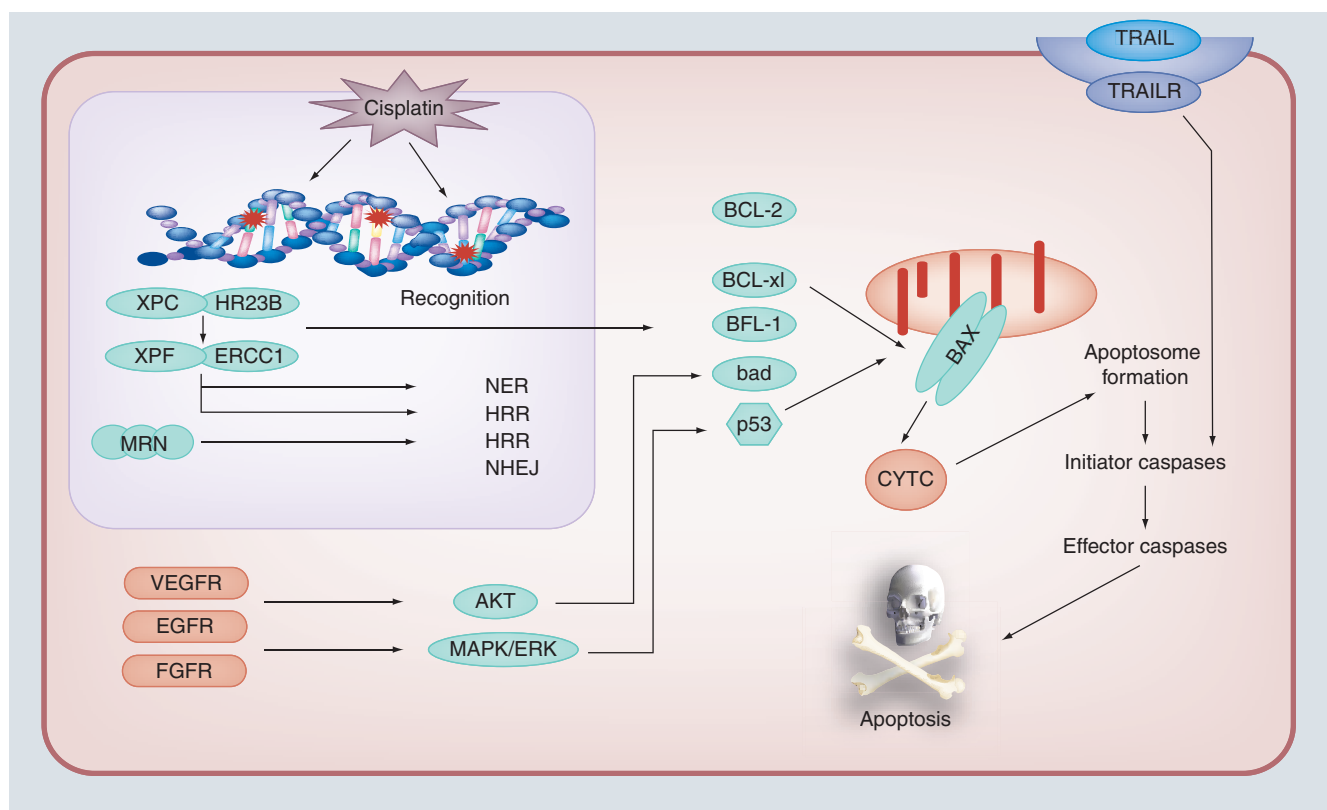


Figure 2. Many proteins involved in the detection and repair of DNA damage caused by cisplatin, or in apoptotic signals arising as a result have been shown to be dysregulated in cisplatin-resistant bladder cancer. These include growth factor receptors such as EGFR, VEGFR and FGFR, and complexes that recognize and repair DNA damage such as the XPC–HR23B complex, the XPF–ERCC1 complex and the MRN complex. Bcl-2 family members (bcl-2, bcl-xl, bfl-1 and bad), which ultimately regulate the release of CYTC from the mitochondria, have also been implicated in the development of cisplatin resistance. Also shown are intercellular survival factors such as Akt and MAPK/ERK kinases, which can become activated via growth factor signaling and contribute to cisplatin resistance. Activation of the extrinsic apoptotic pathway (represented here by the TRAILR) has also been shown to potentiate the cytotoxic effects of cisplatin in resistant cells.
CYTC: Cytochrome C; EGFR: EGF receptor; ERCC1: Excision repair cross complementing; FGFR: FGF receptor; HRR: Homologous recombination repair; MRN: MRE11/Rad50/Nbs1; NER: Nucleotide excision repair; NHEJ: Nonhomologous end joining; TRAIL: TNF-related apoptosis-inducing ligand; TRAILR: TRAIL receptor; VEGFR: VEGF receptor.

Defects in the machinery that responds to cisplatin damage: DNA repair

Increased ability to detect, manipulate and repair damaged DNA has been shown to contribute to cisplatin resistance in many cancer types (FIGURE 2). The nucleotide excision repair pathway (NER) primarily deals with bulky adducts in DNA, and is initiated upon the detection of any adduct which causes a distortion of the DNA helix. Cisplatin adducts are known to distort DNA [65], and as such are able to recruit and activate NER proteins. The reason cisplatin remains such a successful drug for the treatment of testicular tumors is because it expresses low levels of the proteins required for NER [66].

The NER pathway consists of several steps. Once bulky adducts in DNA are detected, a patch of approximately 30 bases is excised by two separate complexes (one 3' to the damage and one 5' to the damage). The gap is then filled by a polymerase (typically polymerase δ or ϵ) using the undamaged strand as a template, and finally the DNA backbone is religated by DNA ligase IV [67]. Of

all the proteins involved in NER, it is the complex made up of the excision repair cross-complementing group 1 protein (ERCC1) and the XPF protein that is most frequently linked to cisplatin resistance in many tumor types [68,69]. The ERCC1–XPF complex is responsible for cutting the DNA backbone in the region 5' to the adduct [70] in the NER pathway, but also appears to play an important role in the resolution of interstrand crosslinks [71], and seems to be especially important in regulating sensitivity to cisplatin adducts.

In UCC, increased ERCC1 expression has been linked with increased tolerance of cisplatin in both cellular studies on cisplatin resistant cell lines [72], and in tumor material, where increased ERCC1 expression correlated with poor survival in patients treated with cisplatin-based chemotherapeutic regimens [34].

XPC, an NER protein responsible for initial recognition and binding of DNA adducts (as part of the XPC–HR23B complex), and also a key intermediate signaling protein between DNA damage and cell cycle checkpoint control and apoptosis [73] is

frequently found to be downregulated in UCC cell lines, and in tumors, where it is associated with cisplatin resistance and increased nuclear translocation of *p53* [74]. Recent studies have highlighted an epigenetic mechanism for XPC downregulation in UCC, with histone deacetylase 4 (HADA4) implicated in XPC silencing, and increased HADA4 in bladder tumors correlating with increased cancer severity [75].

A functioning mismatch repair pathway is required for the detection of cisplatin-induced DNA damage, and disruption of this pathway through either mutation or downregulation (most frequently of *hMLH1*) [76] results in a cisplatin-resistant phenotype in many tumor types, including ovarian, endometrial, gastric and colorectal [77]. In spite of this, very little work has been done on the relationship between mismatch repair and cisplatin resistance in UCC, although, reduced *hMLH1* or *hMLH2* expression has been associated with high-grade tumors and muscle invasive tumors [78].

Other DNA repair pathways

Interfering with the formation of the Mre11/Rad50/Nbs1 complex with a dominant negative *Rad50* mutant has also been shown to sensitize tumor cells to cisplatin chemotherapy [79], suggesting that the two pathways responsible for double-strand break repair, homologous recombination and nonhomologous end joining also play a role in cisplatin resistance, although this has not been demonstrated in UCC.

Defects in the machinery that responds to cisplatin damage: apoptosis

Cisplatin has long been known to primarily elicit its cytotoxic effect through in the initiation of apoptotic pathways in a variety of cell types [80], including UCC [19]. While there is some considerable variation in the exact mechanism through which cisplatin elicits its apoptotic effect, these pathways represent a common site of dysregulation in cisplatin-resistant tumors, and may represent a good target for therapeutic intervention.

Intrinsic apoptosis pathway

The bcl-2 family of pro- and anti-apoptotic proteins are responsible for the collation and transduction of upstream apoptotic signals, and ultimately control the release of cytochrome C from the mitochondria via the intrinsic apoptotic pathway [81], leading to activation of downstream initiator caspases (such as caspase 9) and ultimately effector caspases (such as caspases 3, 6 and 7). Bcl-2, the antiapoptotic founder member of this family, has been shown to be upregulated in cell culture models of cisplatin-resistant UCC [82], and attenuation of this upregulation restored cisplatin sensitivity. A more detailed study in T24 UCC cells and a resistant sub-line showed that in the parental cells, cisplatin was able to initiate mitochondria-dependent apoptosis normally, with the expected increase in membrane associated Bax, and cytosolic cytochrome C. In the resistant line, bcl-2 expression was increased, and as a result cisplatin was unable to elicit this apoptotic response unless bcl-2 expression was decreased using siRNA [83].

In a randomized trial, increased levels of bcl-2 staining in tumors (as measured by immunohistochemistry) clearly identified those patients who responded poorly to neoadjuvant cisplatin treatment following radiotherapy [84].

Replacing the *bcl-2* gene which is lost in the KoTCC UCC cell line increased the cisplatin resistance of cells in culture, decreased apoptosis following cisplatin treatment and rendered xenografts much less responsive to cisplatin treatment [85]. Restoring bcl-2 expression also rendered the cells indolent to apoptosis by adenovirus-mediated *p53* gene transfer.

The mitochondrial protein Smac/DIABLO is known to promote apoptosis through inhibitory interaction with the potent apoptotic inhibitor protein XIAP [86]. In UCC, Smac/DIABLO expression has been shown to inversely correlate with tumor stage and grade, with higher expression also being considered a good prognostic sign based on analysis of postoperative disease-free survival. Cisplatin-resistant UCC cell lines also show low levels of Smac/DIABLO expression compared to parental lines, suggesting that this protein may contribute to drug resistance in UCC [87].

Knockdown of *bcl-2* and *bcl-xl*, another antiapoptotic bcl-2 family member with siRNA has been shown to potentiate the cytotoxicity of cisplatin in a panel of UCC cell lines [88]. The antiapoptotic bcl-2 family member Bfl-1 has also been shown to be upregulated by an NF- κ B mediated mechanism in a cisplatin-resistant UCC cell line, where it increases cisplatin resistance [89]. The use of small-molecule inhibitors of bcl-2 family members to increase the efficacy of cisplatin in cells which generally respond poorly to the drug has been demonstrated in preclinical models of non-small-cell lung cancer [90] and breast cancer [91]. However, in spite of the apparent importance of the bcl-2 family in cisplatin resistant UCC, no similar studies have been reported for this tumor type.

p53

p53 is the most commonly mutated gene in human cancers, and loss of *p53* is associated with a variety of undesirable phenotypic characteristics including failure of apoptotic signaling, and lack of cell cycle checkpoint regulation [92]. Mutations in *p53* have also been linked to cisplatin resistance in many cancer types [93], and in testicular cancer cells, downregulation of *p53* with siRNA has been shown to result in increased cisplatin resistance [94]. The apoptotic effect of cisplatin has been studied in *p53* wild-type and mutant UCC cell lines [19]. In tumors which have either lost *p53* or carry a mutation in *p53*, re-establishment of wild-type *p53* expression has been examined as a possible strategy for reversing the antiapoptotic phenotype and restoring chemosensitivity. Adenovirus-mediated overexpression of wild-type *p53* has been shown to be much more efficient at causing cell cycle arrest and apoptosis in cisplatin-resistant UCC cells compared to their parental lines, alongside an increased *p53* mediated expression of p21 and Bax [95]. And when cisplatin is codelivered alongside adenovirus-mediated *p53* overexpression, a synergistic effect is observed in some UCC cell lines [96]. Attempts to utilize *p53* status to identify those patients that might benefit from adjuvant cisplatin showed initial promise, with an early study identifying UCC patients with *p53* alterations

as being more likely to respond to MVAC chemotherapy [97]. However further clinical trials have failed to back up this observation [98]. A more recent Phase III trial attempting to establish the prognostic value of *p53* alterations has an indicator of response to MVAC chemotherapy failed to confirm any correlation between *p53* status and chemotherapeutic response, but the power of this particular study was hampered by a high patient refusal rate [99].

Microarray studies on T24 and KK47 and their cisplatin resistant daughter cell lines identified the inositol 1,4,5-triphosphate receptor type 1 (IP3R1) mRNA as being consistently downregulated in resistant cells, and as result of short-term cisplatin treatment [100]. IP3R1 is implicated in the regulation of mitochondria-dependent apoptosis through the regulation of cytosolic Ca^{2+} , and exogenous expression of IP3R1 was shown to restore cisplatin sensitivity and cisplatin-induced cleavage of poly(ADP-ribose) polymerase to cisplatin-resistant cells.

The same microarray screen also identified the S100 calcium-binding protein p (S100p) as being consistently downregulated in the cisplatin resistant cell lines [101]. Restoring S100p expression in cisplatin resistant cells restored poly(ADP-ribose) polymerase cleavage and cisplatin sensitivity. Neither of these studies examined clinical material.

Extrinsic apoptotic pathway

In contrast to the intrinsic pathway of apoptosis described above, where apoptotic and survival signals from a variety of sources are collated by the bcl-2 family, leading to mitochondrial cytochrome C release and ultimately cleavage and activation of caspases, the extrinsic pathway provides a mechanism for the transduction of extracellular signals (such as the presence of 'death ligands') to initiate apoptosis in a mechanism that does not involve bcl-2 family members or mitochondria [102]. Ligands capable of initiating apoptosis via the extrinsic pathway include CD95 ligand (CD95L), TNF- α , lymphotoxin- α and TNF related apoptosis inducing ligand (TRAIL). These ligands are detected by transmembrane proteins from the TNF receptor (TNFR) superfamily that contain an intracellular death domain. The receptors corresponding to the ligands described above are CD95 (for CD95L), TNFR1 (for TNF- α and lymphotoxin- α) and TRAILR1 and TRAILR2 (for TRAIL).

These ligands and their receptors have become a recent focus for cancer therapy, in particular TRAIL, which has been shown to induce apoptosis in cancer cells while causing less damage to normal tissue than other death ligands such as the Fas ligand (FASL) [103]. Studies in UCC cell lines and primary cells have shown that FASL and TRAIL are able to potentiate the cytotoxic effect of cisplatin, and critically, that treatment of cisplatin resistant cell lines with either FASL [104] or TRAIL [105] was able to significantly improve the cytotoxicity of cisplatin in these cell lines. Interestingly, sensitization with FASL also caused increased cellular accumulation of cisplatin, suggesting that increased sensitivity may be caused by more than just changes to apoptotic pathways. More recent work has focused on inducing apoptosis using human antibodies that target TRAILR2, such as lexatimumab and mapatumumab. While lexatimumab has been shown to have a synergistic effect with cisplatin in T24

UCC cells [106]; to date, no studies have been conducted with these antibodies in cisplatin resistant UCC cells.

Growth factors & mitogenic signaling

The association between growth factor signaling and the response to cytotoxic agents has prompted a number of studies into the utilization of growth factor receptor inhibitors in combination with cisplatin. Often, this work focuses on the EGF receptor (EGFR), which has been linked to cisplatin resistance in a number of tumor types, either through increased activation of antiapoptotic signaling molecules (such as Akt) [107] or through a direct contribution to repair of cisplatin-induced DNA adducts following nuclear translocation and interaction with DNA-PKcs [108]. In certain UCC cell lines, simultaneous inhibition of EGFR and VEGF using vandetanib causes increased sensitivity to cisplatin [109], including the RT4 line, which generally displays a high level of innate cisplatin resistance [110]. However, this effect was not observed in all UCC cell lines examined. Downregulation of EGFR achieved through transfection with LRIG1 resulted in increased cisplatin-induced DNA damage and apoptosis in a UCC cell line [111].

Members of the FGF receptor (FGFR) family are frequently found to be activated by a mutation in UCCs, and are over-expressed in many UCC cell lines [112,113], where they increase the activation of many survival pathways, such as MAPK/ERK and the deactivation of the proapoptotic protein, bad. Ectopic expression of FGFR in a FGFR-negative UCC cell line increased cisplatin resistance by fourfold [114]. *In vitro* studies have shown FGFR inhibition (with the small molecule inhibitor PD173074) to be extremely effective at potentiating the effect of cisplatin in lung cancer [115], but to date no similar work has been carried out in UCC.

A recent paper on bladder cancer TICs found a cisplatin-resistant subpopulation of CD44 expressing cells within a number of bladder cancer cell lines [25]. These cells also displayed increased activation of the intracellular signaling molecules Akt and ERK. When Akt and ERK signaling was attenuated (through use of an inhibitor of Hsp-90), cisplatin sensitivity was restored, suggesting a potential therapeutic strategy for preventing tumor recurrence by resensitizing bladder cancer TICs to cisplatin.

The observation that transfection of nonmalignant cells with the *h-Ras* or *v-Ras* oncogene could render them resistant to multiple drugs (although not cisplatin) [116] and ionizing radiation [117] lead to a number of studies examining the role of *Ras* in cisplatin-resistant UCC. Genetic analysis of tumor material from 18 patients (ten of whom had received previous cisplatin treatment) revealed only one incidence of a mutated *Ras* gene (from a cisplatin-treated patient) [118], suggesting that this is not a common resistance mechanism in UCC. A study on histocultured T4 UCC cells also showed that overexpression of wild-type or mutated *Ras* offered cells no increased resistance to cisplatin or other cytotoxic agents [119]. A more recent study examining *Ras* family member mutations in bladder cancer found no correlation between mutations in *Ras* family members and recurrence, progression or survival [120].

Mechanisms for acquiring cell change: epigenetic gene regulation

Long-term treatment with cisplatin is known to cause changes in the methylation of histones and DNA. The histone deacetylase inhibitor trichostatin A has been shown to sensitize UCC cells to cisplatin [121], presumably by deacetylating histones, which results in a more relaxed chromatin state in which DNA is more readily accessible to DNA damaging agents [121]. However, epigenetic events underlie changes in the expression of many genes and microRNAs (miRs) and are likely to also contribute to a cisplatin resistant phenotype in a more complex manner than by simply decreasing the accessibility of DNA to cisplatin. The involvement of miRs in the modulation of cisplatin resistance in bladder cancer remains poorly understood, but outside of bladder cancer there are many examples of miRs expression contributing to cisplatin resistance. miR-214 has been shown to target phosphate and tensin homolog in ovarian cancer cells, with an associated increase in cisplatin resistance [122], and the miR200bc/429 cluster has been shown to modulate cisplatin resistance in human gastric and lung cancer cell lines by targeting bcl-2 and XIAP [123]. MicroRNA expression is frequently altered by epigenetic processes in bladder cancer [124,125], and it is likely that epigenetically-regulated changes in miRs expression could contribute to cisplatin resistance in bladder cancer.

Examples of mRNA expression changes that have been shown to be epigenetically regulated in UCC include decreased XPC expression in advanced UCC, which has been shown to be mediated by increased action of histone deacetylases (HDACs; and in particular HDAC4). Inhibition of HDACs using valproic acid increased transcription factor binding to the XPC promoter, increased XPC expression, and increased sensitivity to cisplatin [75]. The DNA demethylating agent 2'-deoxy-5-azacytidine restored hMLH1 expression and cisplatin sensitivity in mouse xenografts of A2780/cp70, an ovarian cancer cell line generated by long-term culture in increasing concentrations of cisplatin [126]. A more recent experiment further increased the cisplatin sensitivity of A2780/cp70 cells *in vitro* and in mouse xenografts using a combination of 2'-deoxy-5-azacytidine, and the HDAC inhibitor belinostat (PXD101) [127]. Despite displaying promise in ovarian cancer, studies on epigenetics in UCC have previously focused on the promise of epigenetic signatures to act as urine-detectable biomarkers [128], and while some effort has been made to use analysis of DNA methylation to discriminate between invasive and noninvasive disease [129], manipulation of the epigenetic status to circumvent cisplatin resistance in UCC has not been reported.

Conclusion

Resistance to cisplatin is a common trait in bladder tumors. At the molecular level this resistance arises *de novo*, with tumorigenesis, or can be acquired through the selection of cisplatin-resistant tumor clones. In general, resistance mechanisms can be divided into those that reduce bioavailability of cisplatin within a cell, and those that attenuate the cell's normal response to cisplatin-induced DNA damage. The expression of key components of these pathways could be used to predict individual tumor response to cisplatin treatment, and therefore personalize chemotherapy use in affected patients. In the long term, strategies to overcome these resistance mechanisms could be used to improve response rates to cisplatin-based chemotherapy.

Expert commentary

Chemotherapeutic resistance is the major hurdle to improving the survival rates from metastatic solid cancers. For example, the cure rates for cisplatin-sensitive tumors, such as testicular cancer, have dramatically improved in recent years. However, the complex and multilayered mechanisms of resistance makes overcoming this cellular phenotype difficult. Specifically, while it has been possible to induce drug sensitivity in resistant cells *in vitro*, this has not yet been reliably demonstrated in patients. However, it should be possible to predict tumor response using the expression of key resistance mediating proteins. This would enable a more rational targeting of cisplatin based regimens to patients most likely to benefit.

Five-year view

In the next 5 years, we will see trials that explore the use of new chemotherapies in bladder cancer. These include traditional nonselective agents, such as novel taxanes and platinum derivatives, and molecular based therapies that target specific defects, as shown by VEGF inhibitors. The heterogeneity of bladder cancer makes it unlikely that one therapy will appear that dramatically improves the prognosis for most tumors within this disease. Whole genome sequencing reports will inform us of the catalog of genetic mutations within these tumors, and hopefully these data will, in the longer term, identify suitable targets.

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Key issues

- Bladder cancer is a common disease characterized by chemoresistance.
- Metastatic disease occurs in up to 50% of patients with advanced disease and responds poorly to treatment.
- Multiagent cisplatin based chemotherapy produces the best response rates.
- Cisplatin resistance is common and may arise *de novo* or be acquired following treatment.
- Molecular mechanisms of cisplatin resistance include reduced intracellular bioavailability (by reduced influx, upregulated efflux mechanism or intracellular sequestration), defective DNA repair or an attenuated apoptotic cascade.
- These molecular characteristics may be produced by genetic or epigenetic mechanisms.

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