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Preclinical Perspectives on Platinum Resistance

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Abstract

In the 30 years since the introduction of cisplatin into the clinic, laboratory studies have provided considerable information as to both how the drug exerts its antitumour effects and how some tumours are, or become, resistant. Once inside a cell, the chlorine groups of cisplatin are exchanged for water (aqua) species, which are more chemically reactive. The intracellular target for cisplatin is DNA, where a variety of adducts are formed, some on the same strand of DNA (intrastrand adducts) and others between strands (interstrand adducts). Of the 4 bases, guanine is the preferred site for binding and the most common adduct involves linkages on 2 adjacent guanines on the same strand of DNA. It remains uncertain which of the various types of adduct is the most important in terms of producing antitumour effects. Resistance to cisplatin has been studied extensively using tumour cells repeatedly exposed to the drug *in vitro*. In these cell models, resistance is generally due to a combination of mechanisms, some resulting in reduced damage to DNA and others following DNA damage. Resistance due to inadequate binding to DNA has been shown to be caused by reduced drug uptake (influx rather than efflux) and inactivation by thiol-containing species such as glutathione and metallothioneins. Resistance occurring post-DNA binding may be due to changes in DNA repair pathways [an increase in nucleotide excision repair (NER) or a loss of DNA mismatch repair (MMR)]. Conversely, the hypersensitivity of some cell lines to cisplatin has been shown to be due to defective NER, through loss or reduced expression of NER proteins such as XPG and XPA. Resistance may also be mediated through alterations in proteins involved in programmed cell death (apoptosis) such as p53 and the BCL2 family. A basic understanding of cisplatin resistance pathways has made a major impact in the development of new platinum analogues capable of circumventing resistance. Examples (which are now undergoing clinical trial) include ZD0473 (which, relative to cisplatin, possesses a reduced reactivity towards inactivating thiol-containing molecules) and the trinuclear platinum BBR3464 (which has markedly different DNA binding properties compared with cisplatin).

Cis-dichlorodiammine platinum (II), or cisplatin, is one of the most commonly prescribed agents for chemotherapy of solid cancers in adults. Although this coordination complex was described in the chemistry literature as early as 1840, its powerful antitumour properties were discovered (by accident) relatively recently by Rosenberg[1] while investigating the effects of electric currents on bacteria in the 1960s.

As with other cytotoxic anticancer drugs, tolerance or resistance of tumours to cisplatin represents a major impediment to successful treatment.

Such resistance is often considered as either intrinsic (i.e. present at the onset of treatment) or acquired (i.e. occurs during chemotherapy). Colorectal and non-small cell lung tumours are typical of those exhibiting intrinsic resistance to cisplatin, whereas tumours in patients with ovarian or small cell lung cancers often develop acquired resistance.

A complete understanding of how cisplatin exerts its antitumour effects (and of why some tumours such as testicular cancer are very responsive while others are intrinsically nonresponsive), plus elucidation of mechanisms of resistance, are pivotal to developing means of combating tumour resistance. During recent years, much insight into mechanisms of resistance to cisplatin has been gained from preclinical laboratory-based investigations using cancer cell lines. This review summarises our present knowledge of cisplatin resistance from such preclinical studies; this knowledge has led to various 'new developments and approaches' discussed in the accompanying paper by Judson and Kelland.[2]

1. Mechanism of Action of Cisplatin

It is generally believed that cisplatin exerts its antitumour properties through binding to DNA, and specific adducts have been identified.[3,4] However, before binding to DNA occurs (and of significance to mechanisms of resistance – see below), loss of the chlorine atoms from the cisplatin molecule is required if antitumour activity is to be expressed. The presence of relatively high chloride concentrations in extracellular fluid (approx 100 mmol/L) suppresses the formation of mono- and diaquo species (in which 1 or both chlorine atoms are replaced by water molecules; the chlorines are often referred to as leaving groups or ligands because they are exchanged, whereas the 2 ammine groups are referred to as carrier ligands and remain attached to the platinum atom even on binding to DNA). In contrast, intracellular chloride concentrations are lower (approximately 4 mmol/L), thereby allowing formation of these species. Since the mono- and diaquo species are far more reactive and better leaving groups than chlorines, this allows reactions with critical cellular nucleophiles, especially DNA (see below).

Cisplatin has been shown to react preferentially with the N7 position of the imidazole ring of guanine (G) or adenine (A) in DNA to form a variety of monofunctional (1 linkage) or, importantly, bifunctional (2 linkages) adducts (fig. 1),[3] which then block replication and/or prevent transcription.

The most common adduct of cisplatin (60 to 65%) involves binding to adjacent deoxyguanosines along the same strand of DNA (the GpG 1,2-intrastrand adduct). Other adducts that have been identified include the ApG 1,2-intrastrand adduct (20 to 25%), and low numbers of GpXpG and ApXpG 1,3-intrastrand adducts (the ApXpG adduct is not shown in fig. 1 as it is a very minor one), monofunctional intrastrand adducts, and G-G

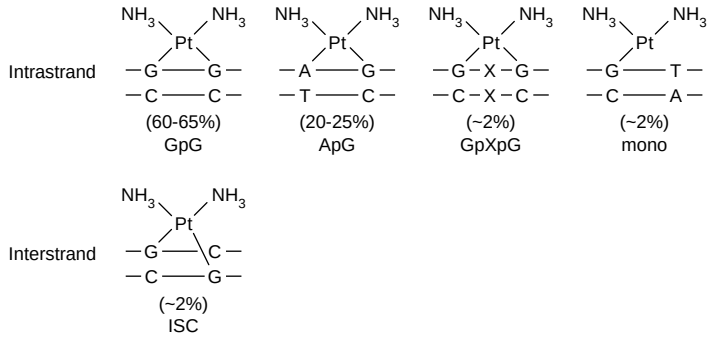

Fig. 1. Binding of cisplatin to DNA: major types of cisplatin adducts (and their incidence). **ISC** = interstrand crosslinks.

interstrand crosslinks (ISCs). Low numbers of DNA-protein crosslinks may also be formed (not shown in fig. 1). Both the GpG and ApG 1,2 - intrastrand adducts unwind DNA by 13°, whereas the GpXpG 1,3-intrastrand adduct unwinds DNA by 23°; bending of the DNA double helix is similar (32 to 35°) for all 3 types of adduct.[4] The high resolution crystal structure of double-stranded DNA containing the GpG 1,2-intrastrand adduct[5] and the nuclear magnetic resonance solution structure of a G-G ISC[6] have been elucidated (see the review by Comess and Lippard[4]).

Further evidence for DNA as the target of cisplatin's antitumour effects is provided by the following observations: (i) cells from patients with diseases in which DNA repair processes are deficient (e.g. xeroderma pigmentosum) are hypersensitive to cisplatin;[7] (ii) correlations have been shown between levels of platinum-DNA adducts in peripheral blood lymphocytes and disease (or toxicity) response in patients receiving cisplatin (or carboplatin).[8]

Despite the elegant studies described above that led to the identification of various DNA adducts, there is still debate as to which adducts are of most importance in mediating tumour cell killing. Some laboratory studies support a role for intrastrand adducts, whereas others indicate that ISCs may be of greater biological significance. Support for the role of the predominant 1,2-intrastrand adducts arises from findings that the inactive *trans*-isomer of cisplatin (transplatin) is sterically unable to form these adducts[9] and that these adducts are relatively poorly removed from DNA, compared with 1,3-intrastrand and monofunctional adducts.[10] On the other hand, although ISCs represent only a small proportion of the overall adducts, studies have shown a relationship between cell killing or resistance and numbers or repair of ISCs[11] (reviewed by Comess and Lippard[4]).

The adducts formed by carboplatin on DNA are essentially the same as those formed by cisplatin, although to obtain equivalent numbers of adducts, 20- to 40-fold higher concentrations are required in cell lines, reflecting the much slower rate of aquation for carboplatin.[12]

2. Resistance to Cisplatin

Laboratory-based studies on mechanisms of resistance have generally used human tumour cell lines (often ovarian cancer) repeatedly exposed to cisplatin *in vitro*. Comparisons at the biochemical and molecular levels can then be made between the resistant subline (resulting from extensive drug exposure) and the unexposed parent line. Typically (and in contrast to the antimetabolite class of cytotoxic drug or drugs that are substrates for the multidrug efflux pump P-glycoprotein), levels of acquired resistance to cisplatin in such experiments are relatively low (often 5- to 10-fold) [for example, see Kelland et al.[13]]. However, this may reflect the fold increase in clinical dose intensity required for cisplatin to overcome acquired resistance. Furthermore, our own studies using a panel of 10 human ovarian carcinoma cell lines showed an initial range in intrinsic cellular sensitivity to cisplatin of more than 100-fold.[14]

In cell lines, 2 broad causes of resistance have been observed: (i) those preventing adequate amounts of drug from reaching and binding to the target DNA; (ii) a failure of cell death to occur after binding of platinum to DNA (fig. 2; for a recent review, see Johnson et al.[15]). These different mechanisms often act together within the same system. Decreased drug transport and increased intracellular drug detoxification/inactivation resulting from increased levels of thiols, especially glutathione or metallothioneins, represent the major causes of inadequate drug concentrations reaching DNA. Once DNA binding has occurred, resistance mechanisms include increased DNA repair of adducts and an ability to tolerate greater levels of DNA damage with a concomitant failure to engage programmed cell death (apoptotic) pathways.

A basic understanding of each of these cisplatin resistance pathways is essential to the development of strategies to combat resistance, either through

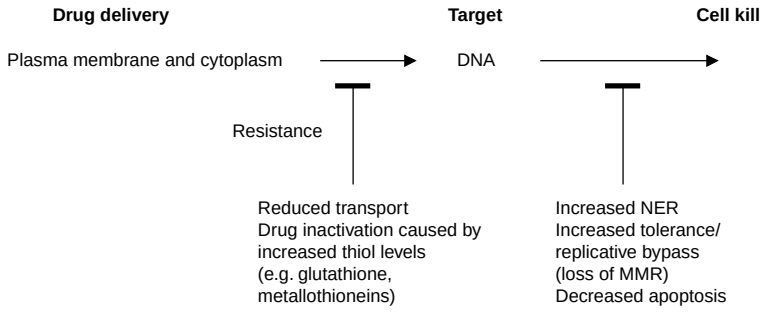

Fig. 2. Major biochemical mechanisms of resistance to cisplatin. Resistance mechanisms may act prior to or after DNA binding. **MMR** = mismatch repair; **NER** = nucleotide excision repair.

the synthesis of improved platinum compounds or through pharmacological modulation.

2.1 Inadequate Binding to DNA

2.1.1 Drug Transport/Uptake

Most *in vitro* models of acquired cisplatin resistance exhibit a decrease in platinum accumulation, typically 2- to 4-fold; it is generally believed that this is due to reduced drug uptake rather than increased drug efflux (the commonly described P-glycoprotein multidrug resistance efflux pump is not overexpressed in cisplatin-resistant tumours).[16,17] However, the mechanism by which cisplatin enters cells is not fully understood; it appears to involve mainly passive diffusion, but with the additional possibility of some facilitated transport, maybe through a gated channel or via a carrier (see reviews by Gately and Howell[16] and Andrews[17]). The uptake of cisplatin is not saturable or inhibited by structural analogues; however, at least a proportion of uptake is energy dependent and can be modulated by pharmacological agents, e.g. the Na^+K^+-ATPase inhibitor ouabain and the membrane-interactive agents amphotericin B (an antifungal) and digitonin.[16,17]

While there have been occasional reports of alterations in levels of proteins in transport-deficient acquired cisplatin-resistant cells, there are no universally accepted platinum transport proteins identified to date. Recently, interest has focused on arsenical transporters[17] and the canalicular multispecific organic anion transporter (cMOAT or MRP2), a member of the ATP-binding cassette family of transport proteins, which is overexpressed in some cisplatin-resistant cells exhibiting a platinum transport defect.[18] Overall, however, the mechanism by which cisplatin enters cells remains rather poorly defined.

2.1.2 Drug Inactivation

Intracellular inactivation of platinum drugs may occur prior to DNA binding through conjugation to thiol-containing species, namely the cytoplasmic tripeptide glutathione or metallothioneins (a class of cysteine-rich, low-molecular-weight isoproteins), and subsequent substitution at the platinum centre. Many platinum-resistant cell lines exhibit relatively high levels of intracellular glutathione compared with their platinum-sensitive counterparts, and a direct interaction between 1 mole of platinum complexed with 2 moles of glutathione has been shown in tumour cells.[19]

Glutathione levels showed a significant correlation with cisplatin sensitivity in our own panel of 8 human ovarian carcinoma cell lines exhibiting a 100-fold range in intrinsic sensitivity (fig. 3), and cells could be sensitised to platinum drugs by depleting glutathione levels with buthionine sulfoximine.[20] Observations such as these, supported by those from many other laboratory investigations, emphasised the importance of thiols in mediating resistance and led to the development of the

sterically hindered platinum drug ZD0473, which is less susceptible than cisplatin to inactivation by thiol substitution and is now undergoing clinical trials (see the accompanying paper by Judson and Kelland[2]). Similarly, increased levels of metallothioneins have been described in at least some acquired cisplatin-resistant cell lines and, moreover, transfection of the human metallothionein MT-II_A cDNA into cells conferred over 4-fold resistance to cisplatin.[21]

2.2 Post-Binding Mechanisms

It is now clear, at least from cell-line studies, that even if sufficient platinum forms adducts on the DNA of a tumour cell, cell death may still not ensue. Various proteins have been described that recognise and bind to platinum-DNA damage. These so-called damage-recognition proteins include XPA-RPA complex, nonhistone chromatin HMG1 and HMG2, human upstream binding factor, histone H1, the TATA-box binding protein TBP, and hMSH2; they may either assist in the removal and repair of DNA damage or, conversely, shield damage from repair proteins.[4,22]

Removal of platinum-DNA lesions is widely believed to occur mainly by nucleotide excision repair (NER), in which an ATP-dependent multiprotein complex removes the damage in the form of a 27- to 29-base-pair oligonucleotide (reviewed by Chaney and Sancar[22]).

There is evidence of increased NER of platinum-DNA adducts contributing to resistance to platinum drugs and, conversely, defective NER contributing to the hypersensitivity of some cell lines.[22] Increased removal of platinum from the total genome of resistant cell lines relative to sensitive parent lines has been observed in several models.[23] Furthermore, NER can occur preferentially in actively transcribed genes relative to the overall genome (transcription-coupled repair).[24] Increased gene-specific repair of cisplatin ISCs has been reported in cisplatin-resistant human ovarian cancer cell lines.[24] Increased levels of RNA encoding the ERCC1 protein, which is involved in NER, have been associated with resistance to cisplatin.[25] Other NER-associated proteins have also been implicated: a defect in the XPG protein caused hypersensitivity to cisplatin in mouse leukaemia cells,[26] and the hypersensitivity of testicular germ cell tumours to cisplatin-induced DNA damage has recently been attributed to reduced XPA protein levels leading to poor damage removal by NER.[27]

These observations of a possible role of increased DNA repair contributing to resistance to cisplatin has led to the following strategies: (i) design of new platinum compounds capable of inducing effects on DNA that are dramatically different from those of existing compounds (e.g. the trinuclear platinum compound BBR3464; see the accompanying paper by Judson and Kelland[2]); (ii) attempts to enhance the therapeutic effect of cisplatin by combination with DNA repair inhibitors [e.g. aphidicolin (an inhibitor of DNA polymerases) and gemcitabine (an inhibitor of DNA polymerase-mediated chain elongation and exonuclease repair)].[28]

Another important group of proteins, the HMG-box proteins, including HMG1 and HMG2, bind selectively to DNA modified by cisplatin but not to that modified by the inactive isomer transplatin.[29] In contrast to the NER proteins, HMG1 has been shown to inhibit the repair of the predom-

Fig. 3. Correlation between intracellular glutathione levels and sensitivity of 8 human ovarian carcinoma cell lines to cisplatin (note that data points for 2 cell lines overlap). From Mistry et al.,[20] with permission from Churchill Livingstone.

inant GpG 1,2-intrastrand cisplatin-DNA adduct by human excision nuclease *in vitro*.[29] A model for the role of the HMG proteins involving shielding of adducts from repair proteins has been proposed.[29]

In many cases, cisplatin resistance appears to be mediated through an increased ability to tolerate drug-induced damage on DNA without NER occurring. Enhanced replicative bypass, the ability of the replication complex to synthesise DNA past a cisplatin-induced adduct, has been shown to occur in some cisplatin-resistant cells.[22,30,31] Although not much is known of the underlying cellular pathways leading to increased tolerance, one component involves dysfunction of a second DNA repair process, mismatch repair (MMR).[22,32,33] This is a recently described, although incompletely elucidated, ATP-dependent repair process involving at least 5 proteins (MLH1, MSH2, MSH3, MSH6 and PMS2) which is responsible for correcting misincorporated nucleotides.[34]

In contrast to NER, where an increase contributes to resistance, it is loss of MMR that has been associated with low level (approximately 2-fold) resistance to cisplatin and carboplatin but, interestingly, not to oxaliplatin or satraplatin (JM-216).[32] In cisplatin-sensitive MMR-proficient cells, futile cycles of excision and resynthesis are thought to cause cell death.[22] Moreover, the hMSH2 protein recognises cisplatin-induced GpG-1,2-intrastrand adducts on DNA.[35] Furthermore, some acquired cisplatin-resistant tumour cell lines have lost MMR activity, primarily because of defects in the hMLH1 subunit.[22,32,33] Loss of hMLH1 has been shown to be due, in some cases, to promoter methylation, leading to the proposal that tumours could be treated with the combination of cisplatin and the methylation inhibitor azacitidine (5-azacytidine).[33] MMR defects in hMutSα (a heterodimer of hMSH2 and hMSH6) or hMutLα (a heterodimer of hMLH1 and PMS2) have been shown to contribute to an increase in replicative bypass of cisplatin adducts.[30] Drug resistance in MMR-deficient tumours is then thought to occur by prevention of futile cycles of translesion synthesis and mismatch correction.

Various other genes and proteins have been implicated in determining cellular sensitivity and resistance once platinum has bound to DNA, thus providing further confusion as to how cisplatin kills cells.[36] Important among these are genes involved in mediating programmed cell death (the *p53* tumour suppressor gene, the *bcl2* gene family, and c-*myc* and *ras* genes).[37] Cells exposed to cisplatin (and other platinum drugs) show the morphological and biochemical characteristics of apoptosis, namely chromatin condensation, membrane blebbing and DNA fragmentation.[38]

The possible importance of the *p53* gene (one of the most commonly altered genes in human cancers) in determining the sensitivity of tumour cells to cisplatin (and other DNA damaging drugs) is revealed in studies using the National Cancer Institute drug screening panel of 60 cell lines. Cells with mutant *p53* sequence were more resistant to cisplatin than those possessing wild-type p53 function.[39]

The *bcl2* family of genes encodes a group of proapoptotic (e.g. BAX, BAK, BAD) and antiapoptotic (e.g. BCL2, BCL-X_L) proteins, which form homo- and heterodimers with one another. It has been proposed that relative levels of pro- and antiapoptotic proteins may function as a cell survival/cell death rheostat to influence sensitivity and resistance to apoptosis-inducing drugs such as cisplatin.[40] However, in our panel of human ovarian carcinoma cell lines, high levels of the antiapoptotic protein BCL2 conferred a trend toward increased sensitivity, not resistance, to cisplatin.[41]

3. Conclusions

In most preclinical models of cisplatin resistance (either acquired or intrinsic), multiple mechanisms appear to operate. A combination of effects, resulting in a reduction in the amount of platinum binding to the target DNA and insufficient cell death once binding has occurred, is common. However, there is no consensus as to which types of effects may be most important. Thus, if these cell

line observations are relevant to platinum-based chemotherapy in man, then combating resistance to cisplatin represents a challenging prospect (see the accompanying article by Giaccone[42]).

Nevertheless, the elucidation of these major biochemical mechanisms of resistance has been crucial in providing a basis for the development of platinum-based compounds capable of circumventing cisplatin resistance. Such agents, some of which are currently undergoing clinical trial (and are discussed in another chapter[2]), include the diamminocyclohexane platinums such as oxaliplatin,[43] the sterically hindered drug ZD0473[44,45] and trinuclear platinum complexes (such as BBR3464).[46]

A consequence of the multiple mechanisms of resistance is that combination therapies targeting different steps may be of clinical value. For example, a platinum analogue such as ZD0473, which presents a steric hindrance to thiol activation, may be usefully combined with a DNA repair inhibitor such as gemcitabine to circumvent at least some of the post-binding mechanisms, or even with another platinum drug possessing different DNA binding properties (such as BBR3464). Preclinical drug combination studies in cell lines or mice bearing human tumour xenografts may be useful in guiding clinical trials, particularly with respect to drug scheduling and prediction of toxicities.

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