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Chapter 1

Mechanisms of Cisplatin Resistance: DNA Repair and Cellular Implications

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Abstract

Cisplatin (cis-diamminedichloroplatinum (II)), is a platinum based chemotherapeutic employed in the clinic to treat patients with lung, ovarian, colorectal or head and neck cancers. Cisplatin acts to induce tumor cell death via multiple mechanisms. The best characterized mode of action is through irreversible DNA cross-links which activate DNA damage signals leading to cell death via the intrinsic mitochondrial apoptosis pathway. However, the primary issue with cisplatin is that while patients initially respond favorably, sustained cisplatin therapy often yields chemoresistance resulting in therapeutic failure. In this

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chapter, we review the DNA damage and repair pathways that contribute to cisplatin resistance. We also examine the cellular implications of cisplatin resistance that may lead to selection of subpopulations of cells within a tumor. In better understanding the mechanisms conferring cisplatin resistance, novel targets may be identified to restore drug sensitivity.

Keywords: Cisplatin resistance, interstrand cross-links, DNA repair, tumor-initiating cell

Introduction

Cisplatin, (cis-diamminedichloroplatinum (II), or CDDP) was first approved for clinical use in 1978 for treatment of testicular and bladder cancers [1]. The observed anti-tumor effects have led to the use of cisplatin as a frontline chemotherapeutic in the treatment regimens for patients with lung, ovarian, colorectal or head and neck cancers [1-3]. While still clinically relevant today, cisplatin has also spawned the platinum-based drug derivatives carboplatin and oxaliplatin. Patients treated with these platinum-based drugs display early therapeutic success with disease regression or a halt in further progression. As with many chemotherapeutic options, adverse events exist for cisplatin with observed kidney, peripheral nerve and inner ear toxicity [4, 5]. Yet the primary issue with cisplatin therapy is that despite initial therapy successes, many patients eventually develop resistant disease [6-8]. Notably, cisplatin refractory disease is also cross-resistant to carboplatin [9]. Given that cisplatin based therapies remain the sole therapeutic option in certain circumstances [10], it is key that the underlying mechanisms potentiating resistance are uncovered.

Mode of Action

As a platinum based compound, cisplatin is inert but is activated through a series of aquation reactions. The aquation process involves the spontaneous substitution of one or both cis-chloro groups with water molecules in the cytoplasm [11, 12]. This process makes aquated cisplatin highly reactive toward intracellular nucleophiles [13]. It is this process that yields the anticancer effects of cisplatin through reactions within the cytoplasm and

nucleus. Within the cytoplasm, cisplatin reacts with nucleophiles such as methionine, metallothioneins, reduced glutathione (GSH) and proteins via cysteine residues. In this way, cytoplasmic cisplatin may act like a molecular sponge for reduced species and push a cell towards oxidative stress. Recent evidence that enucleated cytoplasts are responsive to cisplatin further highlights the cytotoxic effects of the cytoplasmic cisplatin module [14, 15]. However, these cytoplasmic reactions may also serve to inactivate cisplatin through antioxidant systems [7].

The best characterized mode of action for cisplatin is via its reactivity with DNA. In the nucleus, aquated cisplatin interacts with high affinity toward nucleophilic N7-sites of purine bases to form interstrand and intrastrand DNA cross-links [16]. However, the cytotoxic effects of nuclear cisplatin are largely caused by the intrastrand DNA cross-links which account for 90% of cisplatin-induced DNA lesions [17, 18]. These lesions distort the DNA structure allowing multiple DNA damage signals to be initiated [19]. One of the initiated signals involves the nucleotide excision repair (NER) complex. This system recognises the distorted DNA and attempts to remove adducts to enable cell survival [20]. Other DNA damage signals involve the mismatch repair (MMR) and base excision repair (BER) systems. Herein, the components of these complexes sense the DNA lesions and commence the signals instructing cell fate [21-23]. Hence, the cell will initially attempt to repair the cisplatin-induced DNA lesion through one of the listed DNA repair mechanisms (see above). However, the cytotoxicity of cisplatin relies on overwhelming incidence of the adducts, as well as the high proliferative rate of the tumor. This leaves the repair of DNA largely unattainable. As a result, cell death will ensue predominantly via the apoptotic cascade.

The key link between DNA damage and apoptosis involves the ataxia telangiectasia mutated (ATM)-and Rad3-related (ATR) kinase and the checkpoint kinase 1 (CHK1). Cisplatin-dependent sequential activation of ATR and CHK1 leads to activation and stabilization of tumor suppressor p53 through phosphorylation at serine 20 [24-27]. p53 is the central mediator of cisplatin-induced apoptosis. In cisplatin-sensitive cells, p53 induces apoptosis via both nuclear and cytoplasmic mechanisms. In the nucleus, p53 transcriptionally up-regulates genes involved in cell cycle arrest and DNA repair [28] as well as multiple pro-apoptotic genes including *NOXA* [29] and *Bax* [30]. These pro-apoptotic factors are involved in initiation of the complex intrinsic mitochondrial apoptosis pathway [31]. Cisplatin induces the p53-mediated localization of both NOXA and Bax to the mitochondria yielding the release of cytochrome *c* which activates caspase-dependent apoptosis [32, 33].

In particular, the balance of cell fate is hinged upon the ratio of Bax with the anti-apoptotic Bcl-2. In cisplatin-sensitive cells, Bcl-2 is lost via proteolysis enabling activation of the apoptosis cascade [34, 35]. Interestingly, cisplatin is also reported to induce apoptosis via mitochondria independent mechanisms, but nonetheless utilizing the caspase 8-caspase 3 pathway via p53 [36, 37].

Mechanisms of Cisplatin Resistance

It is clear that multiple mechanisms are active in sensitizing cells to cisplatin. Alterations to any of these pathways underlie the cisplatin resistant phenotype observed in the clinic. As such, cisplatin resistance can ensue from (i) reduced intracellular cisplatin uptake, (ii) increased efflux of cisplatin from the cell as well as (iii) an enhanced ability of cells to repair cisplatin-induced DNA distortions. Herein, describing the mechanisms relating to cisplatin uptake and drug efflux go largely beyond the scope of this chapter but are adequately reviewed elsewhere [38, 39].

In the following sections we describe three potential mechanisms through which cisplatin resistance may occur, with an especial emphasis on processes downstream of DNA lesion formation. In this sense, we focus on the deregulation of DNA damage repair and suppressed apoptotic signalling as means of developing cisplatin resistance. Also, the role of tumor clonality in cisplatin resistance is discussed.

Deregulation of DNA Damage Repair

As discussed in previous sections, the nucleophilic attack of aquated cisplatin on purine bases can result in the formation of three major lesion types (i) bulky cisplatin adducts on singular purines, (ii) intrastrand cross-links between bases on the same strand and (iii) interstrand cross-links between bases on opposing strands (Figure 1) [40]. In each instance, the cell will initially respond by launching a DNA damage response in an attempt to remove the lesion. This response will vary depending on the nature of the adduct, as well as on the cell cycle phase. For instance, while nucleotide excision repair (NER) is likely the major pathway for removal of cisplatin bulky adducts and intrastrand cross-links outside of S phase of the cell cycle [41], translesion synthesis and/or repair by homologous recombination may represent significant pathways during replication [42]. Furthermore, while

intrastrand cross-links may be processed efficiently by translesion repair alone during replication, the overcoming of interstrand cross-links probably involves proteins of the Fanconi anemiahomologous recombination pathway (see below) [43]. While most likely representing minor repair roles, base excision repair and mismatch repair of cisplatin adducts may represent important mechanisms of 'sensing' cisplatin-mediated DNA damage [44].

Here we describe the known DNA repair pathways involved in the recognition and repair of cisplatin adducts, and discuss some of the recognised mechanisms through which resistance may occur.

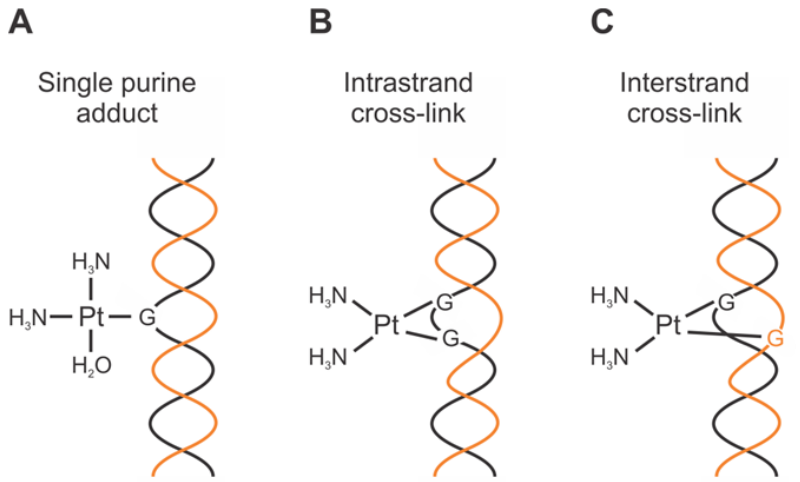

Figure 1. Cisplatin-induced DNA adducts. The predominant DNA adducts caused by cisplatin include (*A*) bulky cisplatin adducts on singular purines, (*B*) intrastrand cross-links between bases on the same strand and (*C*) interstrand cross-links between bases on opposing strands.

Mismatch Repair and Base Excision Repair

Mismatch repair (MMR) is an important cellular pathway in the correction of endogenous polymerase-mediated replication errors [22, 23]. Additionally, MMR may be involved in the repair of small DNA lesions e.g. those caused by alkylating agents [45]. In the earliest instance, base-mismatches are recognized by one of two MutS heterodimers; MutSα (MSH2/MSH6), which binds singular base mismatches or insertions, or MutSβ (MSH2/MSH3), which recognizes larger distortions [46-48]. These complexes are then able to recruit one of three MutL complexes, MutLα (MLH1/PMS2), MutLβ (MLH1/PMS1), or MutLγ (MLH1/MLH3), which, in concert with proliferating cell nuclear

antigen (PCNA) and replication factor C (RFC), are able to unwind the helix at the damage site [49, 50]. Exonuclease 1 (EXO1) may then degrade a section of the damaged strand, including the mismatch/lesion, which is replaced by the concerted efforts of DNA polymerase and DNA ligase [51].

Although MMR is not able to repair cisplatin adducts, the MutSβ heterodimer has been documented to interact with cisplatin intrastrand cross-links [52, 53]. Here it is able to recruit MutL complexes, which, being unable to repair the damage, initiate pro-apoptotic signals; this process represents a key aspect of cisplatin-mediated toxicity [54-56]. It is therefore not surprising that these events are often suppressed in cancer cells which are resistant to cisplatin. In particular, the MutS components MSH2, MSH3, and MSH6, as well as MLH1 of the MutL complex, are often under-expressed or mutated in cancer cells displaying platinum resistance [57-61].

The pathway of base excision repair (BER) has also recently been suggested to impact on cisplatin-lesion repair [62]. This pathway primarily functions in the removal of small base damages, primarily as a result of base oxidation or alkylation [21]. DNA adducts are initially recognized by a specific DNA glycosylase, the identity of which depends on the type of base damage [63]. In the case of cisplatin interstrand cross-links, a new model has suggested that adjacent 'extra-helical' bases that have been disrupted by the crosslink may be subject to glycosylase recognition. This is predicted to initiate a pathway of non-productive processing in which the disrupted base is deaminated by the glycosylase, leaving an abasic residue [62]. The phosphodiester backbone is then most likely cleaved by AP endonuclease 1 (APE1), and the residue replaced by DNA polymerase-mediated strand synthesis; the cisplatin cross-link will however remain. While this pathway does not seem to initiate apoptotic signaling akin to that stimulated by MMR, the localization of BER proteins may preclude other DNA repair proteins from binding the site of damage, preventing the proper clearance of the lesion [44]. In this model, and similarly to MMR, BER seems to function in non-reparative cisplatin adduct recognition; unlike MMR however, BER protein localization is likely to suppress apoptosis induction. In agreement, the increased expression of APE1, DNA polymerase β and human 8-oxoguanine DNA glycosylase (hOGG1) has been attributed with cisplatin resistance, presumably through suppression of this signalling [64-66].

Nucleotide Excision Repair

Nucleotide excision repair (NER) represents an important pathway for the repair of a large number of structurally varied DNA adducts [67]. These

lesions are seemingly recognized as a result of their disruption to base pairing, and the resulting helical distortion, more so than by their specific chemical nature [68]. In keeping, NER has been found to be the major pathway involved in the removal of cisplatin adducts, and as such, is up-regulated in many platinum-resistant tumors [69, 70].

In the initial stages of NER, damage is recognized by a complex composed of the proteins XPC and hHR23B, which appear to initiate unwinding on either side of the lesion [71]. This is further stimulated by the helicase activity of the TFIIH transcription factor, as well as by XPA and replication protein A (RPA) [72], which help expand the bubble. The exposed adduct may then be excised by the concerted efforts of two major nucleases, XPF-ERCC1, which cleaves the phosphodiester backbone 5` to the lesions, and XPG, which cleaves on the 3` side [73, 74]. These events allow for the removal of an approximately 24-32 nucleotide signal stranded oligomer containing the lesion [75]. This gap may then be replaced by the activities of DNA polymerases δ and ε, which use the intact complementary strand as a template, followed by closing of the phosphate backbone by DNA ligase 1-mediated ligation [76].

While the above seems to reflect the NER response to singular cisplatin adducts and intrastrand cross-links [77-79], the response to interstrand cross-links is apparently more complex. This is most likely due to the damage caused to both strands of the helix, such that neither can function as an appropriate repair template. While a large proportion of cisplatin interstrand cross-links are repaired by translesion repair and homologous recombination during S phase [43, 80, 81], there is compounding evidence that an NER-translesion repair pathway may exist during G1/G0 [82-84]. Following recognition of DNA interstrand cross-links, the NER nucleases are suggested to cleave the phosphodiester backbone on either side of the adduct as per above [85], leaving the ‘excised’ oligomer covalently bound to the opposing strand through the base crosslink. The cell is then able to bridge the gap by action of a specialized class of DNA polymerases known as translesion polymerases (discussed further below), which are able to insert bases opposite the damage site, although with an increased likelihood of introducing base mismatches when compared to those involved in replication [82, 86, 87]. The remaining ‘excised’ oligomer, and the section of the opposing strand to which it is bound, is then presumably cleaved and resolved by a second NER excision event [88].

Due to the apparent capacity of the NER system to remove each form of cisplatin adduct, it is not surprising that an upregulation of this pathway is

frequently observed as a means of conferring cisplatin resistance. As may be expected, the expression of multiple NER repair proteins has been found to negatively correlate with cisplatin sensitivity, including that of ERCC1 [89-92], XPA [91, 93], and XPC [94]. In the case of XPA, expression levels appear to be regulated by the hypoxia-inducible factor-1α (HIF-1α) [93], an essential transcription factor in the response to hypoxic conditions. Contrasting this, low-level expression of HIF-1α has been correlated with better overall survival in patients with small-cell lung cancer who receive platinum-based chemotherapy, indicating HIF-1 α-mediated NER de-regulation may not be a key mechanism of resistance [95]. In addition, p53-mediated transcriptional regulation of a number of NER proteins also seems to promote resistance to a number of cross-linking agents [96]. For example, acquired cisplatin-resistance in a subset of malignant melanoma cells has recently been attributed to p53-dependent transcriptional up-regulation of XPC [94]. Further, the translational synthesis of XPA, XPC, hHR23B and the RPA subunit RPA32 has also been found to be negatively regulated by eukaryotic initiation factor-3a (eIF3a), the expression of which has been positively correlated with sensitivity to cisplatin [97, 98]. As opposed to deregulated NER protein expression, platinum resistance has also been correlated with gene polymorphic status. Of note, SNPs in *ERCC1* have received recent attention, with a number of studies suggesting an association between some of these variants and cisplatin sensitivity [91, 99-101].

Classical Translesion Repair

Although the NER pathway is able to remove most forms of bulky adduct, in some instances, lesions are able to sustain into S phase. This is especially true of interstrand cross-links, which for this reason, were originally thought only to be processed by homologous recombination. Two possible explanations have been put forward regarding this inefficiency: (i) that interstrand cross-links are not always efficiently recognized when occurring in highly condensed chromatin [43], and (ii) that some of these damage events may be incompletely removed due to their chemical complexity [102].

By any means, the sustainment of bulky DNA adducts into S phase represents a considerable obstacle for the migrating replicative fork, as the processive δ and ε DNA polymerases are not able to insert opposite damaged bases [103]. Additionally, the covalent crosslinking of opposing DNA strands may prevent helicase-mediated unwinding ahead of the fork [104]. In either instance, replication is forced to stall until such time that the adduct is repaired or bypassed.

The pathways of translesion repair and homologous recombination are currently recognized as the major cellular strategies involved in re-starting stalled replicative forks. In the majority of instances, the choice between the two pathways appears largely governed by the mono-ubiquitination of the PCNA sliding clamp, which seems to promote the recruitment of a number of translesion polymerases. The overcoming of interstrand cross-links seems the main exception to this rule (discussed in subsequent sections), where cross-over between the two pathways is apparent [81, 105, 106].

The translesion polymerases largely consist of proteins from the Y-family of polymerases (polη, ι, κ and Rev1), as well as polζ of the B-polymerase family. In response to fork stalling, these polymerases may be recruited to sites of damage, largely as a result of Rad18/Rad6 mediated-mono-ubiquitination of PCNA [107]. PCNA is a central protein in the recruitment and activity of both replicative and translesion polymerases, although its mono-ubiquitination seems to increase its affinity for the later [108]. Although each of the translesion polymerases initially seems to interact with PCNA, 'competition' for bypass of the lesion is resolved by the differing substrate specificities of each enzyme, such that the adduct is overcome by the most appropriate polymerase(s). In the bypass of cisplatin bulky adducts and intrastrand cross-links, the translesion polymerases Polη [109-111], Polζ [112, 113], and Rev1 [114-116] seem to play the most important roles.

Consistent with our discussion on the NER pathway, an upregulation of the translesion synthesis pathway has been associated with cisplatin-resistance. Of particular note, expression of Rev3, the catalytic subunit of Polζ, has been correlated with cisplatin-resistance in head and neck [117] and cervical tumors [118]. Furthermore, suppression of Rev3 in cisplatin-resistant cells in culture [117] and *in vivo* [119] resulted in re-sensitization to platinum therapy, suggesting this as a driving factor of resistance. While increased clearance of cisplatin adducts is likely the major outcome of increased Rev3 expression, the inherent mutagenicity of Polζ translesion replication has also been cited as a potential mechanism of resistance development [120, 121].

In addition to Polζ, a positive correlation between Polη protein expression and platinum resistance has also been observed [122, 123]. Polη represents the major polymerase in the bypass of GpG intrastrand cross-links, where it is able to bypass the lesion in an essentially error-free manner [124]. For this reason, and unlike Polζ, the apparent cisplatin-resistance phenotype afforded by Polη is likely to result purely from increased repair capacity, and not from increased mutagenic rate.

Homologous Recombination at the Replication Fork

As discussed above, cisplatin induces interstrand cross-links which block the DNA replication fork by preventing DNA strand separation. To enable cell survival, restoration of replication by removal and repair of the interstrand cross-link is required. As described in detail below, this process involves interplay between factors involved in homologous recombination double strand break repair as well as members of the Fanconi anemia pathway [43]. Indeed, cells deficient in homologous recombination proteins (such as BRCA1 or BRCA2) or Fanconi anemia proteins, exhibit an increased sensitivity to cisplatin [125].

However, these factors alone are insufficient for DNA repair with other repair pathways, such as base and nucleotide excision repair, or translesion repair, required to complete DNA repair. Despite the contribution of additional pathways, homologous recombination is the primary pathway through which DNA cross-links are removed to allow replication to restart.

Homologous recombination is a series of interrelated pathways that repair DNA double strand breaks in an error free manner by using DNA strand invasion and template directed DNA synthesis. As shown in Figure 2, the initial step of repair requires the processing of the break to generate a 3' overhanging tail of ssDNA that is initially coated and protected by the ssDNA binding protein RPA [126]. Subsequently, BRCA2 (FANCD1) functions to remove and replace RPA with RAD51 to allow formation of the pre-synaptic filament [127].

This RAD51 nucleofilament is involved in the search for and annealing with the homologous template; a process called strand invasion. The newly formed structure is termed a D-loop, where the invading strand is elongated using the invaded duplex DNA as a template [128].

The capture and annealing of the second end of the double strand break is catalyzed by the RAD52 protein and generates a structure called double Holliday junction [129].

This structure of interlaced DNA strands is dissolved in a non cross-over product by a complex formed by the DNA helicase Bloom (BLM) and the topoisomerase TOPOIIIα, ultimately achieving an error- free repair of the DNA break [130].

Repair pathways are activated when replication forks collide with an interstrand cross-link during S phase, blocking the replisome (Figure 3). When one fork encounters such a block, the spontaneous collapsing of the fork generates a one-ended double-strand break.

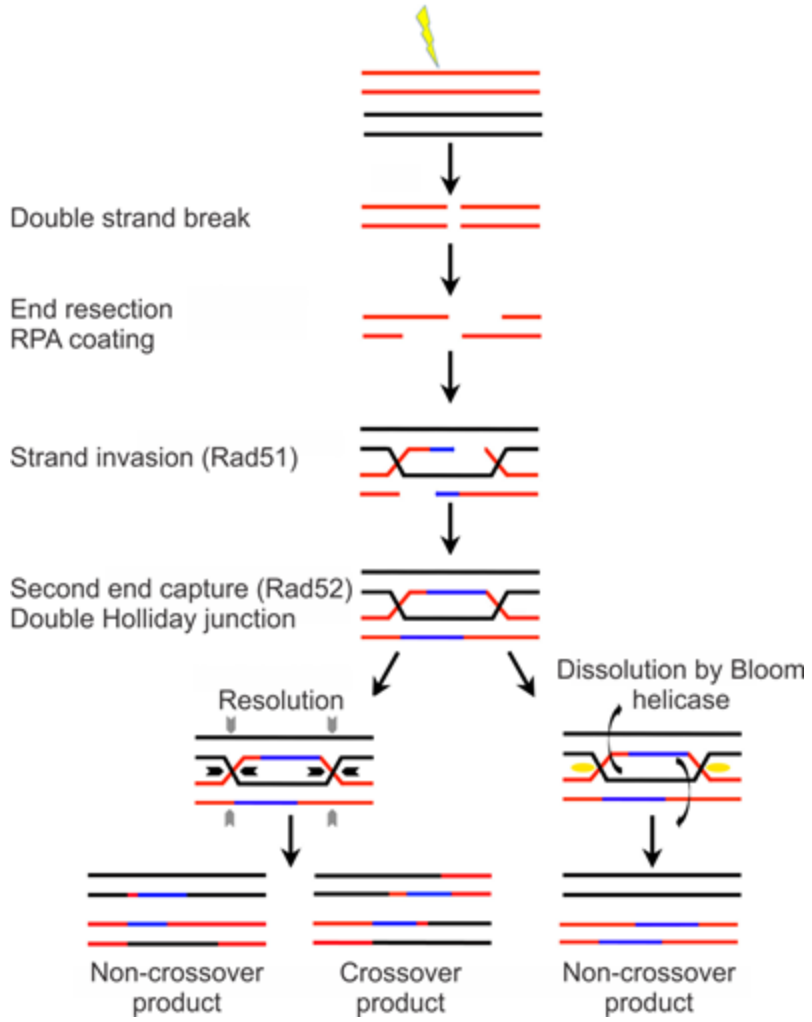

Figure 2. Repair of DNA double strand breaks (DSB) by homologous recombination. Following a DNA double strand break, the DNA is recessed forming a 3' overhang, allowing coating of the ssDNA with RPA. RPA is replaced by Rad51 initiating the search and annealing with a homologous template (strand invasion) to create a D-loop. A double Holliday junction is then formed when the opposite DNA strand from the break anneals to the D-loop strand. The Holliday junction is resolved either creating a chromatid crossover product or non-crossover product.

The repair of the collapsed fork then requires the invasion of the broken strand into the intact double strand of DNA, this pathway is mediated by RAD51, which catalyses the strand invasion, similar to the one use in

homologous recombination (see above). The interstrand cross-link is cleaved by the MUS81-EME1 endonuclease complex allowing the bypass of the lesion [131]. The broken leading strand is then extended by translesion synthesis polymerases, completing the homologous recombination. Following this process, replication can then restart.

As shown in Figure 3, such collapses can be avoided by FANCM-mediated fork regression and fork stabilization. Following unhooking of the cross-linked DNA, this stabilized structure allows the leading strand of synthesized DNA to be extended by the translesion synthesis machinery. The one-ended double strand break is then processed and repaired by homologous recombination followed by replication restart. In this setting, the protein FANCM, in complex with FAAP24 and the MHF1/2 histone-fold dimer, recognizes the helix distortion caused by the interstrand cross-link [132]. The specificity of binding for the stalled fork seems to be provided by FAAP24. Although FANCM is a helicase, the protein is not able to catalyze strand separation. Rather, FANCM acts as a dsDNA translocase by remodelling the fork to permit processing and extensive fork regression [133]. At the same time, FANCM/FAAP24 activate an interstrand cross-link specific ATR-CHK1 mediated response, by its ability to generate a ssDNA stretch that is rapidly coated by RPA [134].

Independent of this signalling function, FANCM/FAAP24 recruits the Fanconi anemia core complex to the stalled fork. This complex, which is formed by eight Fanconi anemia proteins (A/B/C/E/F/G/L/M), prevents the fork from collapsing [132, 135]. Together with the activation of the ATR pathway, the recruitment of the Fanconi anemia core complex promotes the monoubiquitination of the FANCD2/I complex, allowing its localization to the chromatin [136].

This modification is catalized by the E3 ligase activity of FANCL and is essential for the scaffolding role of FANCD2, which then binds the DNA repair protein FAN1 [137]. The nuclease FAN1 performs a nucleolytic incision on the 5’ side of the lesion. The 3’ side is cleaved by the MUS81/EME1 nucleases, finally promoting the unhooking of the cross-link, generating a double strand break and allowing the translesion synthesis polymerases to bypass the lesion. In addition to the recruitment of FAN1, the FANCD2/I complex enables recruitment of the proteins BRCA2 (FANCD1), FANCJ and PALB2 (FANCN) to the site of damage. This process leads to the formation of a RAD51 coated presynaptic filament initiating homologous recombination and replication restart.

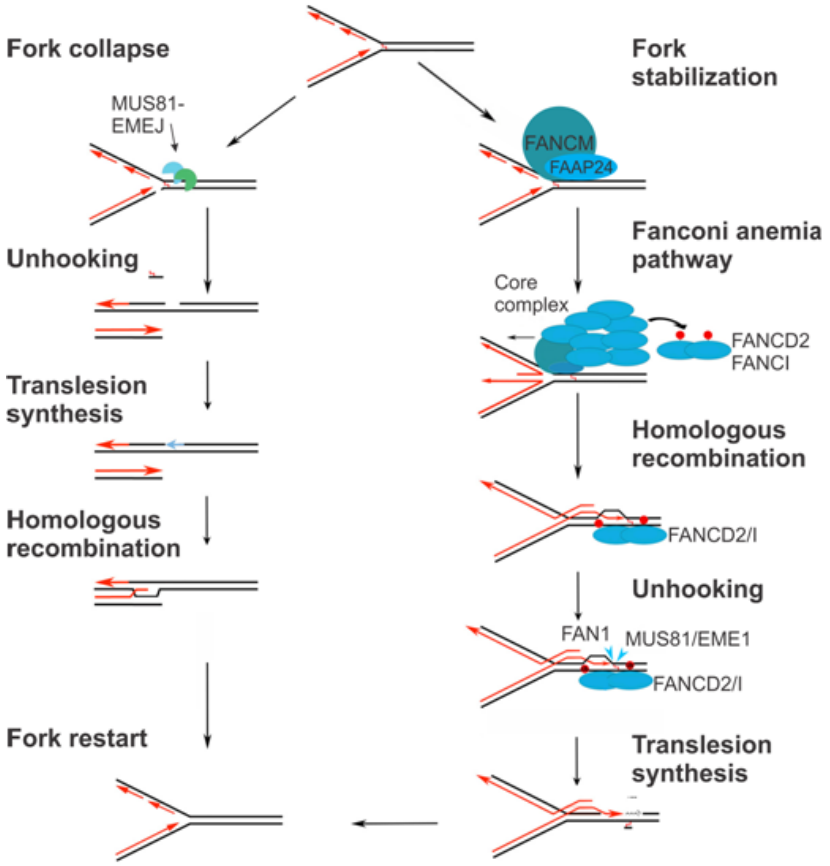

Figure 3. Speculative model of how Fanconi anaemia-associated proteins repair interstrand cross-links during DNA replication. Cisplatin induces an interstrand cross-link preventing progress of the DNA replication fork. Blockade of the replication fork causes either the fork to collapse (left) or the fork to stabilise (right). Replication can only restart after unhooking (removal) of the cross-linked DNA and DNA repair either by homologous recombination (left) or by the Fanconi anemiapathway (right).

An alternative scenario is when two replication forks, arriving from opposite directions, collide at the interstrand cross-link. In this instance, one fork stops 24 nucleotides ahead of the interstrand cross-link while the other fork stops only one nucleotide away from the damage. Each replication fork may be stabilized in a process similar to that of single fork stabilisation. In this way, adduct removal takes place by cutting the leading strand template at either side of the cross-link. A complex series of ligations and elongations of the DNA strands results in the formation of a typical Holliday junction

structure. Replication can then restart after resolving the Holliday junction via a complex of multiple factors including BLM helicase, topoisomerase TOPIIIa, RMI1, RMI2 and RPA [138]. Interestingly, although BLM helicase has been shown to have multiple roles in cisplatin removal by the homologous recombination pathway, it has not yet been linked to acquired cisplatin resistance.

Fanconi Anemia and Homologous Recombination Proteins in Cisplatin Resistance

Fanconi anemia is a rare genetic disorder characterized by congenital abnormalities and progressive failure of bone marrow leading to increased susceptibility to haematological cancers [139]. Cells from these patients are highly sensitive to agents inducing interstrand cross-links, resulting in chromosomal aberrations [140]. As such, cisplatin therapy is not able to be used to treat these patients. Our knowledge of the Fanconi anemia and homologous recombination pathways in these patients is aiding out understanding of how these pathways contribute to cisplatin resistance in tumors. For example, hypersensitivity of some ovarian cancer cells to cisplatin is linked with disruption of the Fanconi anemia pathway. In this setting, cells lacking the monoubiquitinated form of FANCD2 are sensitive to cisplatin [141]. This is due to methylation of the FANCF gene lowering its expression. [141]. As part of the Fanconi anemia core complex, FANCF is involved in FANCD2/FANCI ubiquitnation. Interestingly, in cells acquiring cisplatin resistance, FANCF function is restored by endogenous gene demethylation, allowing monoubiquitination of FANCD2 [141]. Sensitivity of other ovarian cancer cells to cisplatin has also been linked with tumors exhibiting *BRCA2* gene mutations [142]. In these cells, prolonged exposure to cisplatin is reported to induced secondary intragenic mutations in *BRCA2* leading to restoration of the reading frame and rescue of gene function, ultimately resulting in cisplatin resistance [142]. A similar mechanism of cisplatin resistance was observed in ovarian cancer cells exhibiting *BRAC1* gene mutations [143]. Secondary mutations in *BRAC1* also yielded cisplatin resistance [143]. Together, these studies highlight the contribution of proteins involved in the Fanconi anemia and homologous recombination pathways to cisplatin resistance.

Suppressed Apoptosis as a Mechanism of Cisplatin Resistance

As discussed above, cisplatin-induced distortion of DNA structure initiates DNA damage signals that link with induction of the apoptotic signal

cascade. However, in chemotherapy tolerant cells, failure to induce apoptosis following cisplatin treatment is a documented mechanism conferring drug resistance. A central player in this process is p53. Dysfunction of p53 is a key mechanism of apoptosis evasion with p53 inactivation through gene mutation present in over half of all neoplasms [144-146]. Consistently, in both *in vitro* and *in vivo* settings, cells harboring p53 defects have been linked with poor cisplatin sensitivity [147-149]. In the clinic, ovarian and testicular cancer patients exhibiting expression of wild type p53 are also likely to respond favorably to cisplatin therapy with improved 5-year survival rates compared with patients presenting with mutant p53 [150, 151]. The escape of apoptosis may indeed be context dependent, as there are several reports suggesting that mutant p53 expressing cells remain sensitive to cisplatin [149, 152-154]. Nonetheless, the *in vitro* evidence indicates that in cells expressing mutant p53, the predominant mechanism of cisplatin resistance is via an inability of p53 to initiate the downstream apoptotic cascades [155-159].

Cells expressing wildtype p53 are also capable of developing cisplatin tolerance and resistance. In these instances, mechanisms exist to attenuate p53 function other than by gene mutation. One such example in cisplatin resistant cells is the aberrant overexpression of MDM2, a negative feedback regulator of p53, and potential downregulation of the MDM2 inhibitor, $p14^{ARF}$ [160-163]. In another instance, oncogenic protein phosphatase magnesium-dependent 1 (PPM1D) is reported to confer cisplatin resistance by attenuating both p53 and CHK1 function through dephosphorylation and preventing their stabilization [164]. Mislocalization of p53 may also contribute to cisplatin resistance. For example, cytoplasmic mislocalized p53 is reported to interact with and inhibit caspase-9, allowing evasion of apoptosis [165]. In addition, mutational ablation of p53 nuclear localization signals, leading to cytoplasmic sequestration of p53, is also emerging as a mechanism for conferring cisplatin resistance [166, 167]. In this way, preventing p53 localization to the nucleus enables the manifestation of cisplatin resistance through an inability to transcriptionally upregulate the pro-apoptotic machinery.

Despite the dependence on p53 to decide cell fate, aberrant expression of factors downstream of p53 in the apoptotic cascade is also capable of influencing cisplatin resistance. For example, overexpression of the apoptotic inhibitors survivin and XIAP has been linked with enhancing cisplatin resistance by impacting caspase function [168, 169]. Similarly, overexpression of anti-apoptotic factors Bcl-2, Bcl-X_L and MCL-1 in cisplatin resistant cells is observed *in vitro* and in multiple cancers [170-173]. This is associated with the *in vitro* downregulation of the pro-apoptotic factors BAX and BAK in

cisplatin resistance [31, 174, 175]. Together, these reports highlight the complex regulation of the apoptotic cascade involved in yielding cisplatin resistance.

Tumor Clonality in Cisplatin Resistance

In the previous sections, we have reviewed the complex DNA repair mechanisms and signalling pathways contributing to cisplatin tolerance and resistance. In the following sections, we describe the (i) cellular implications of cisplatin resistance within a tumor and (ii) review the current strategies to profile the genomic and epigenetic aspect to cisplatin resistant disease.

The spatial heterogeneity of tumors is a consequence of tumor evolution [176]. Selection pressures enhance survival of cell sub-populations and allow clonal relationships to form within a tumor. Next generation sequencing has exemplified the clonal nature of tumors within a number of primary cancers including breast [177, 178], prostate [179, 180], renal [181] and pancreatic [182, 183] cancers. While clonal relationships develop during tumor evolution as a result of oncogenic mutations [184] or genetic drift, recent evidence suggests that cisplatin also contributes to tumor clonality. For example, *in vitro* and *in vivo* cisplatin treatment of lung cancer cell lines and patient tissue caused an enrichment of 'stem-like' $CD133^+$ cells [185, 186]. Similarly, in tumors collected from ovarian cancer patients, primary platinum treatment enriched for cells expressing the stem-like markers aldehyde dehydrogenase, CD44 and CD133 [187].

An emerging paradigm in chemotherapy is that cells with stem-like characteristics, also termed tumor-initiating cells, are chemotherapeutic resistant [188, 189]. In particular, cisplatin-induced enrichment of these stem-like cells has been reported to confer drug resistance. For instance, Barr et al. showed that cisplatin-induced apoptosis in $CD133^+/CD44^+$ cells enriched from lung cancer cells was markedly reduced versus the bulk population of cells [190]. Moreover, these cells displayed a reduced uptake of cisplatin together with a reduced formation of DNA cross-links [190]. Consistently, in a separate study, $CD133^+$ enriched lung cancer cells with sphere forming characteristics had reduced cisplatin-induced PARP-dependent apoptosis [191]. Most interestingly, implantation of tumor-initiating spheres from H125 lung cancer cells also exhibited a cisplatin resistant phenotype *in vivo* [191]. Similar observations have shown that tumor-initiating cells isolated from other NSCLC cell lines also exhibit a cisplatin refractory phenotype [185, 192, 193].

Moreover, in ovarian cancer, tumor-initiating spheres exhibited cisplatin and paclitaxel resistance as mediated by the stem cell factor receptor c-Kit [194]. Indeed, these reports point to the likelihood that drug resistant tumor clones exhibiting stem-like characteristics contribute to recurrent disease.

How tumor-initiating cells remain refractory to chemotherapy is an active area of research but the mechanisms remain elusive. However, as discussed earlier in this chapter, evidence suggests that tumor-initiating cells also possess the means to evade cisplatin-induced apoptosis and confer drug resistance through the DNA damage repair pathways [195]. For example, a recent report demonstrates that DNA double strand break repair, as assessed by γH2AX foci, is enhanced in cisplatin resistant NSCLC cells enriched for tumor-initiating cell markers $CD133^+/CD44^+$ and ALDH [190]. Also in NSCLC cells, sphere forming tumor-initiating cells resistant to cisplatin were found to have higher basal levels of γH2AX yet diminished ATM phosphorylation and monoubiquitination of FANCD2 [191]. Pharmacological inhibition of ATM in NSCLC tumor-initiating cells before and during drug treatment also conferred cisplatin resistance [191]. However, further investigations are required to characterise the DNA repair mechanisms that contribute to cisplatin resistance in tumor-initiating cells.

A number of other cell signaling pathways appear to be active during the formation of cisplatin resistant tumor clones. One such pathway is the notch signaling pathway. Classically, notch signaling is particularly prevalent during development but also in stem cells [196, 197]. Activation of the membrane receptor notch by its mammalian ligand, Jagged, induces proteolysis of notch and translocation of the intracellular domain to the nucleus to modulate transcription [198]. Notch expression is associated with cisplatin resistance in a number of tumors including cervical cancer [199], head and neck squamous cell carcinoma (HNSCC) [200] and ovarian cancer [201]. More strikingly, notch signalling also contributes to acquiring cisplatin resistance. Inhibition of notch signalling by pharmacological or knockdown approaches sensitized drug resistant HNSCC cells [200, 202], cervical cancer cells [199], non-small cell lung cancer cells [203] and ovarian cancer cells [204] to cisplatin. Importantly, notch signalling is also a key feature in mediating cisplatin-induced enrichment of $CD133^+$ cell subpopulations from lung adenocarcinoma cells [186]. Hence, the notch pathway presents as an interesting therapeutic target in cisplatin resistant cells.

Another pathway of interest in cisplatin resistance is the WNT/β-catenin signalling pathway. The WNTs couple to a complex network of receptors and co-receptors to activate canonical (β-catenin dependent) or non-canonical (β-

catenin independent) downstream signaling [205]. These signaling pathways contribute to mammalian development and stem cell proliferation [206]. Recently, the canonical WNT/β-catenin is also suggested to contribute to cisplatin resistance. In drug resistant triple negative breast cancer, WNT signalling via the receptor FZD8 is reported to contribute to *in vivo* tumor-initiating cell enrichment induced by the combinational treatment of cisplatin with TRAIL [207]. Similarly, in chemoresistant ovarian cancer, activation of the WNT/β-catenin signalling pathway by c-Kit confers cisplatin resistance in tumor-initiating cells by upregulating the ABC drug transporter ABCG2 [194]. Notably, both β-catenin and ABCG2 are overexpressed in chemoresistant and recurrent ovarian carcinoma [187, 208]. Further indication that WNT/β-catenin signalling may contribute to drug resistance is the observation that the potent WNT signalling inhibitor Dkk-1 is downregulated in cisplatin resistant glioma cells [209] and head-neck cancer cells [210].

Genomic and Epigenetic Profiling of Cisplatin Resistant Disease

It is clear that cisplatin induces tumor clonality by enriching for drug resistant tumor-initiating cells via several signalling pathways. However, the genetic basis that drives these mechanisms remains unknown. In chemotherapeutic refractory disease, use of genomic profiling has identified mutations and pathways that drive resistance [211, 212]. For example, secondary mutations in KRAS are detected in the blood of colorectal cancer patients undergoing anti-EGFR therapies that confer drug resistance [213, 214]. As discussed earlier in this chapter, secondary mutations on the *BRCA1* and *BRCA2* genes have been discovered in response to cisplatin therapy [142, 143]. However, only a handful of studies have investigated the wider genetic component to cisplatin refractory cancer. In one such study, sequential exome sequencing of circulating tumor DNA from an ovarian cancer patient over a period of 2 years identified an increase in a truncating mutation of the retinoblastoma 1 (RB1) gene [215]. Interestingly, loss of RB1 function is linked with a varied chemotherapeutic response in breast and prostate cancers [216]. In addition, genetic profiling of copy number variants (CNVs) has identified genetic changes in response cisplatin [217]. In particular, 70 cisplatin-associated CNVs were identified (66 as biallelic) of which 87% were deleterious. Of interest, one of the duplicated CNVs predicted the expression

of glutathione reductase [217], which is associated with cisplatin sensitivity [218].

In addition to genomic changes in cisplatin resistance, it is also clear that epigenetic changes may contribute to drug resistance. Changes in methylation of CpG islands in cisplatin resistant cells leading to gene downregulation are well documented [219-222]. One example is methylation-dependent downregulation of *MLH1* expression, which is a component of the MMR DNA repair pathway [223]. Cisplatin-induced loss of *MLH1* expression occurs in 25-30% of ovarian cancer patients and is associated with poor patient prognosis [223, 224]. Use of genome-wide DNA methylation profiling is also revealing potential epigenetic drivers of cisplatin resistance. For example, use of demethylating agents and microarray analysis identified several hundered genes downregulated in isogenic cisplatin resistant cancer cells [219]. In this study, bisulfite sequencing also confirmed that 14 genes were hypermethylated in resistant cells but that expression of six of these genes (*SAT*, *C8orf4*, *LAMB3*, *TUBB*, *G0S2*, *MCAM*) were induced following cisplatin treatment in sensitive cells but not resistant cells [219]. More recently, use of isogenic sensitive and resistant ovarian cancer cells has also identified a large array of hypermethylated genes [222]. In this study, of the identified 4092 hypermethylated genes, only 245 genes were silenced while demethylation of 41 of these genes led to cisplatin resensitization [222]. Interestingly, 4 of these 41 genes (*ARMCX2*, *COL1A1*, *MEST*, and *MLH1*) were found to be hypermethylated following acquisition of cisplatin resistance in TICs from IGROV1 ovarian cancer cells [222].

The state of chromatin is also implicated in cisplatin tolerance via histone deacetylases (HDAC). HDACs modify histones enabling chromatin maintenance and transcription. HDAC4 silencing is reported to sensitize drug resistant ovarian cancer cells to cisplatin via a mechanism dependent on STAT1 [225]. Consistently, early use of HDAC inhibitors in drug resistant ovarian cancer cells resensitized cells to cisplatin through an induction of the intrinsic apoptosis pathway independent of p53 [226]. Interestingly, use of HDAC inhibitors has also led to the re-expression of several methylated genes [227, 228], notably *MLH1* in both *in vitro* and *in vivo* settings [229]. Importantly, modified chromatin structure has also been linked with drug resistant TICs. Sharma et al. reported that HDAC inhibitors also yield cisplatin sensitivity in tumor-initiating cells but that chromatin state is altered by the histone demethylase RBP2/KDM5A/Jarid1A in these cells [230].

Together, these studies point to a genetic and epigenetic component to cellular responsiveness to cisplatin. However, further efforts are required to

genotype the tumor clones resistant to cisplatin therapy. In particular, monitoring disease before and during cisplatin therapy will elucidate further genetic and epigenetic components as well as pathways leading to chemotherapy refractory disease.

Conclusion

How tumors affect cisplatin resistance is the key issue facing healthcare providers. As discussed in this chapter, there are distinct mechanisms yielding drug resistance through the DNA damage and repair pathways as well as via attenuation of the apoptotic cascade. These signalling pathways are seemingly active within the context of tumor, with subpopulations of cells intrinsically refractory to cisplatin therapy. It is important to note that cisplatin resistance is no doubt multifactorial. Therefore, understanding the multiple mechanisms that are active in cisplatin resistant cells is key to preventing survival of intrinsically resistant cells as well as cells that may gain drug tolerance. In this way, generation of therapeutics and strategies targeting these mechanisms of resistance may restore cisplatin sensitivity and circumvent drug resistance.

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