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Biochemical Modulation of Cisplatin Mechanisms of Action: Enhancement of Antitumor Activity and Circumvention of Drug Resistance

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1. Introduction

Biochemical modulation is the manipulation of cellular biochemical pathways by chemical agents to produce selective enhancement of the efficacy of an antitumor drug.[1] Since the introduction of cisplatin (*cis*-DDP), *cis*-diamminedichloroplatinum(II), into general oncology practice, the studies dealing with the molecular mechanism of action of the drug have provided considerable information as to how cisplatin induces its antitumor effects. The biochemical mechanisms of cisplatin cytotoxicity involve the binding of the drug to DNA and non-DNA targets and the subsequent induction of cell death through apoptosis, necrosis, or both.[2] In fact, it has been proposed that a functional cooperativity between these two forms of cell demise is regulated by the intracellular redox potential generated by the pyridine nucleotide pool (NAD^+/NADH and $NADP^+$/NADPH ratios) and the cellular free energy available from the ATP/ADP ratio.[3,4] Cisplatin is highly effective in the treatment of testicular and ovarian cancers and is also employed for treating bladder, cervical, head and neck, esophageal, and small cell lung cancer.[5] However, some tumors such as colorectal and nonsmall cell lung cancers have intrinsic resistance to cisplatin, while others such as ovarian or small cell lung cancers develop acquired resistance after the initial treatment.[6] Biochemical studies have not clearly established the molecular bases of resistance to cisplatin in any type of cell, but, at least, they have identified several mechanisms that can contribute to this phenomenon. Resistance to cisplatin is generally multifactorial and has been shown to be due to reduced drug accumulation, inactivation by thiol containing species, increased repair/tolerance of platinum−DNA adducts, and alterations in proteins involved in apoptosis.[7,8] One strategy to overcome cisplatin resistance is to design platinum complexes that specifically deal with some or even all of the above-mentioned resistance mechanisms. However, after more than 30 years of intensive research from the discovery of the antitumor activity of cisplatin, no more than 30 compounds have shown enough pharmacological advantages relative to cisplatin to be tested in clinical trials.[9] Moreover, only four

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platinum drugs are currently registered for clinical use (marketed drugs).[10] Among the registered platinum drugs (see Figure 1), carboplatin, [*cis*-diammine-1,1′-cyclobutane dycarboxilate platinum(II)], is less toxic than cisplatin but possesses the same spectrum of antitumor activity. The only registered platinum drug that has consistently demonstrated antitumor activity against cisplatin resistant tumors such as colorectal cancers is oxaliplatin, [*trans*-L-1,2-diaminocyclohexaneoxalatoplatinum(II)].[11] However, there have been recently reported several novel classes of platinum complexes able to circumvent cisplatin resistance in preclinical or even clinical studies including *cis*-Pt(II) compounds with planar ligands, *trans*-Pt(II) and *trans*-Pt(IV) compounds, and polynuclear platinum complexes.[9]

Taking into account the above-mentioned considerations, there is little doubt that the search for novel platinum compounds able to circumvent cisplatin resistance has proven to be a difficult task. An alternative way based on the use of biochemical modulation strategies directed to circumvent cisplatin resistance could enhance the antitumor activity of cisplatin improving the outcome of cancer patients. So far, an increasing number of drugs centered on the biochemical mechanisms of modulation of cisplatin resistance have been identified.[6] The present review gives an update of the state-of-the-art of the biochemical modulation of cisplatin mechanisms of resistance and looks for future directions of research on this important issue.

2. Biochemical Mechanisms of Action of Cisplatin

It is generally accepted that binding of cisplatin to genomic DNA (gDNA) in the cell nucleus is largely responsible for its antitumor properties.[2] The damage induced upon binding of cisplatin to gDNA may interfere with normal transcription and/or DNA replication mechanisms. Eventually, these disruptions in DNA processing would trigger cytotoxic processes that lead to the death of the cancer cell. However, it is known that cisplatin forms a high amount of adducts in mitochondrial DNA (mtDNA) lacking histones.[12] Moreover, mitochondria are unable to carry out nucleotide excision repair (NER), a major pathway for removing cisplatin−DNA adducts.[6] So, it should not be ruled out the possibility that mtDNA may also be an important pharmacological target for cisplatin. In any event, previous to cisplatin binding to genomic or mitochondrial DNA a loss of chloride groups is required. However, the

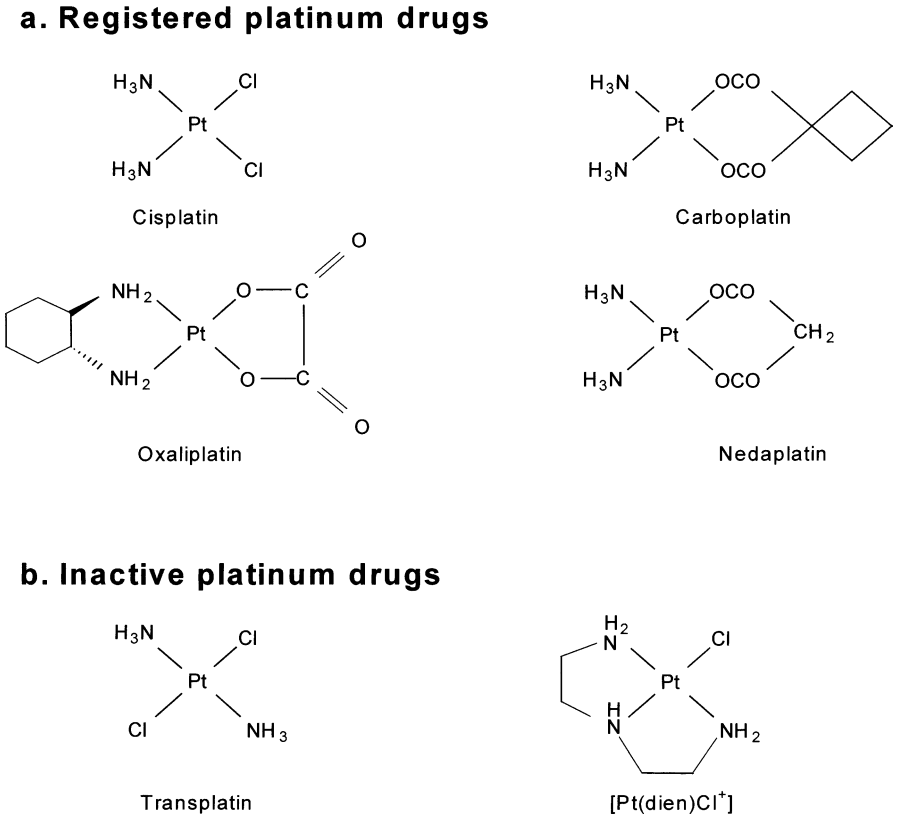


Figure 1. (a) Structures of the four platinum antitumor drugs currently registered for clinical use (marketed drugs) and of (b) two biologically inactive platinum compounds.

high chloride concentration in extracellular fluids (≈100 mM) suppresses the formation of mono- and diaquo *cis*-Pt(II) species in which one or both chloride groups are replaced by water molecules. In contrast, within the cell, the chloride concentration ranges between 2 and 30 mM. In this range of Cl^- concentrations, the hydrolysis of *cis*-DDP occurs efficiently so that one or both chloride leaving groups are replaced by water molecules, allowing the formation of aquo species. The final result is the formation of the $[Pt(H_2O)_2(NH_3)_2]^{2+}$ cation. This diaquo species is very reactive toward nucleophile centers of biomolecules because H_2O is much better leaving group than Cl^-.[13]

2.1. Binding of Cisplatin to DNA Targets

The N7 atoms of the imidazole rings of guanine and adenine located in the major groove of the double helix are the most accessible and reactive nucleophilic sites for platinum binding to DNA.[14] The reaction of cisplatin with DNA may lead to the formation of various structurally different adducts. Initially, monofunctional DNA adducts are formed, but most of them further react to produce interstrand or intrastrand cross-links, which then block replication and/or prevent transcription.[15] It has been found that 60−65% of adducts formed by cisplatin are 1,2-d(GpG) intrastrand cross-links and 20−25% 1,2-d(ApG) intrastrand cross-links. Minor adducts, each accounting for a few percent, include 1,3-intrastrand cross-links and interstrand cross-links.[16] DNA−protein cross-links have also been reported to be induced by *cis*-DDP.[17] Both the 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links unwind DNA by 13°, while the 1,3-d(GpXpG) intrastrand cross-links unwind DNA by 23°. Interestingly, however, bending of the DNA double helix is similar (32−35°) for these three types of intrastrand adducts.[18] There is still debate as to which types of cisplatin−DNA adducts are the most important in mediating the cytotoxicity of *cis*-DDP. Support for the role of the major 1,2-intrastrand adducts in cisplatin-induced tumor cell killing arises from the fact that due to steric reasons, the inactive trans isomer of cisplatin, *trans*-DDP or transplatin (see Figure 1), is unable to form these adducts. In fact, *trans*-DDP mainly forms 1,3-intrastrand and interstrand cross-links.[19] Moreover, it has been found that the 1,2-intrastrand adducts are less effectively removed from DNA by repair enzymes than 1,3-intrastrand adducts.[20,21] Further support for 1,2-intrastrand cross-links as the main adducts responsible for the antitumor activity of cisplatin came from the discovery that some HMG (high mobility group) domain proteins specifically recognize this type of DNA adduct (see Figure 2). So, it has been proposed that specific HMG proteins may be involved in the cellular processing of the 1,2-intrastrand cross-links formed by *cis*-DDP.[22] However, the possible importance of minor adducts, as interstrand and DNA−protein cross-links in the mechanism of cytotoxic activity of cisplatin, should not be ruled out. In fact, some studies have shown a relationship between cell killing or resistance and numbers or repair of interstrand and DNA−protein cross-links.[23] It should be pointed out that interstrand cross-links of *cis*-DDP induce striking distortions on DNA with bending of

Figure 2. Ribbonlike diagram showing the structure of domain A of HMG1 domain protein bound to a cisplatin 1,2-d(GpG) intrastrand adduct on a DNA double helix stretch (taken from ref 12). The HMG domain specifically recognizes the kink formed by 1,2-intrastrand adducts on DNA double helix.

the helix axis toward the minor groove (20−40°) and a large DNA unwinding (≈80°).[24]

2.2. Binding of Cisplatin to Non-DNA Targets

One aspect of the biochemical mechanism of action of cisplatin that has been very little studied is the degree of contribution of targets other than DNA to the cytotoxic effects of the drug. In fact, it is known that only 5−10% of covalently bound cell-associated cisplatin is found in the DNA fraction, whereas 75−85% of the drug binds to proteins.[25,26] In addition, before *cis*-DDP accumulates in the cell, it may bind to phospholipids and phosphatidylserine of the cell membrane.[27] In the cytoplasm many cellular constituents that have soft nucleophilic sites such as cytoskeletal microfilaments, thiol-containing peptides, and proteins, and RNA react with cisplatin.[28] Due to the strong reactivity of platinum compounds against S-donor molecules, the most important non-DNA target of *cis*-DDP is probably the tripeptide glutathione (GSH), which is present in cells at high concentrations (0.5−10 mM).[6] GSH and other thiol-containing biomolecules such as metalothioneins (MT) bind rapidly to platinum and this binding has primarily been associated with negative phenomena, including the development of resistance and toxicity. However, it has been hypothesized that cisplatin binding to GSH perhaps may serve as a drug reservoir modulating the kinetics of DNA platination.[29] On the other hand, *cis*-DDP may affect the activity of enzymes, receptors, and other proteins through binding to sulfur atoms of cysteine and/or methionine residues and to nitrogen atoms of histidine residues. The resulting functional protein damage may also contribute to the biochemical mechanism of cisplatin cytotoxicity. For instance, binding of *cis*-DDP to methionine 1 (met1) and/or histidine 68 (his68) of ubiquitin may inhibit the ubiquitin−proteasome pathway of selective degradation of cellular proteins, which might induce cytotoxic processes.[30] Moreover, it has been recently reported that *cis*-DDP, besides inhibiting the in vitro chaperone activity of heat shock protein 90 (Hsp90), efficiently and selectively blocks its C-terminal ATP binding site.[31]

2.3. Biochemistry of Cisplatin-Induced Cell Death Pathways

It is generally accepted that futile attempts to repair cisplatin-induced DNA damage may finally result in the triggering of apoptosis.[32] Apoptosis, also called "programmed cell death" or "cell suicide", is considered a controlled pathway that requires ATP (adenosine triphosphate) and de novo protein synthesis. There is, moreover, experimental evidence indicating that the protein damage caused by cisplatin, rather than DNA damage, plays a role in triggering apoptotic pathways.[33] It is known that some types of cancer cells when exposed to *cis*-DDP insult show internucleosomal DNA degradation in approximately 180 base pair fragments, blebbing of the cell surface and cell shrinkage. All these features are consistent with apoptosis as a mode of cell death.[34] Besides, it is also known that, in other cell lines, particularly those with resistance to the drug, cisplatin produces characteristic features of necrosis, which is considered a mode of cell death due to general cell machinery failure.[35−37] Moreover, it has been reported that in the same population of cisplatin-treated cells, necrotic and apoptotic cell death may take place together.[38]

Apoptosis and necrosis have been usually considered two separate modes of cell death, not only morphologically but also mechanistically.[39] In the 1980s, necrosis was considered the mode of cell death induced by DNA-damaging anticancer agents because of the activity of poly(ADP−ribose) polymerase (PARP).[40] PARP is activated by the DNA strands breaks caused by some anticancer agents, including cisplatin, and cleaves the glycolytic coenzyme NAD^+ (nicotinamideadenine dinucleotide), provoking the formation of poly(ADP−ribose) moieties (ADPR). Depletion of NAD^+ inhibits glycolytic production of ATP with subsequent ATP depletion leading to necrotic cell death.[3] By the 1990s, it was thought that most clinically effective anticancer agents that bind to DNA kill cancer cells by apoptosis.[41] The apoptotic process is generally divided in three different stages.[2] The first one is an initiation phase, in which a stimulus is received followed by engagement of any one of several possible pathways that respond to the stimulus. The second one is an effector phase in which all the possible initiating signals are integrated and a decision to live or die is made. The last one is a common irreversible execution phase in which some proteins autodigest and DNA is cleaved by an endonuclease. *Bcl-2* is an oncogene that appears to be at the convergence of many apoptotic pathways and the ratio of Bcl-2 to Bax proteins at the effector phase might be the final determinant as to whether a cell enters the execution phase.[42] *Bax* is a gene which encodes a dominant inhibitor of *Bcl-2*.[43] A general property of the execution phase of apoptosis is the

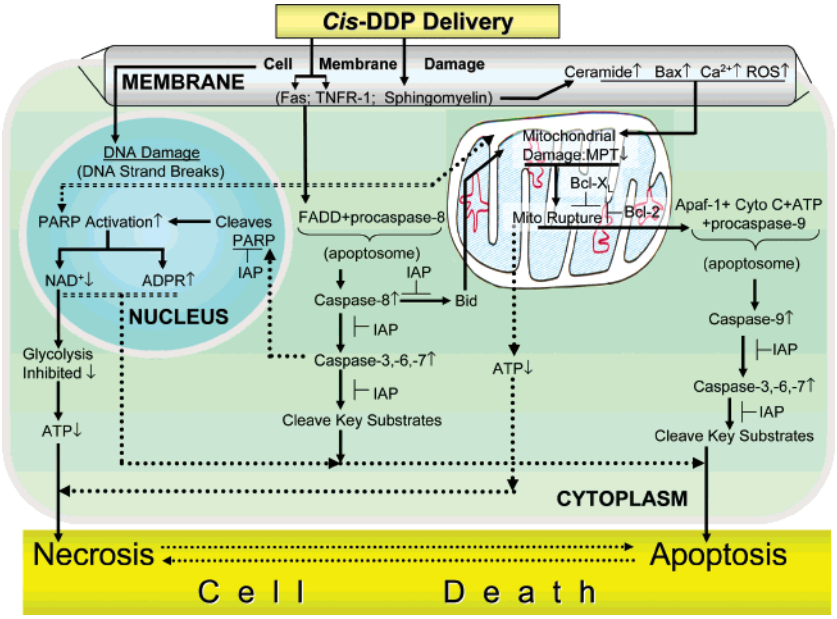


Figure 3. Schematic overview of the proposed biochemical pathways of cell death induced by *cis*-DDP showing the interconnections between apoptosis and necrosis (discontinuous arrows). DNA strand breaks activate poly(ADP−ribose) polymerase (PARP), which cleaves NAD^+ and provokes the formation of poly(ADP−ribose) moieties (ADPR). The result is a decrease in NAD^+ with a concomitant fall of glycolysis and subsequent ATP depletion so that cell death by necrosis takes place. In contrast, if ATP levels are enough to sustain survival, caspase-3-6-7 cleaves PARP, necrosis is blocked, and apoptosis occurs. If PARP cleavage is prevented, the continued activity of PARP leads to enhancement of both necrosis and apoptosis. Apaf-1, apoptotic protease-activating factor-1; Bid, a type of proapoptotic protein; FAAD, Fas-associated death domain; Fas, cell surface membrane receptor; TNFR-1, tumor necrosis factor receptor; IAP, inhibitor of apoptosis; PAR, poly(ADP−ribose); ROS, reactive oxygen species; Cyto *c*, cytochrome *c*; Mito; mitochondrial; MPT, mitochondrial permeability transition.

specific degradation of a series of proteins by the cysteine aspartate-specific proteinases (caspases). Caspases are activated when an apoptotic stimulus induces the release of cytochrome *c* from mitochondria.[44] It has been recently found that mitochondria play a central role in apoptosis. As depicted in Figure 3, cisplatin DNA damage induces a fall in the mitochondrial permeability transition (MPT).[45] Subsequently, the MPT fall releases factors that facilitate the rupture of mitochondria such as reactive oxygen species (ROS), Bax, and Ca^{2+}.[46] Mitochondrial rupture releases cytochrome *c* and procaspase-9 that bind to cytosolic Apaf-1 and ATP in an apoptosome complex, leading to the activation of caspase-9. Activated caspase-9 induces other caspases interactions, resulting in activation of caspase-3, caspase-6, and caspase-7 with the subsequent cleavage of key substrates.[47] The final outcome is the dismantling of the cell by formation of apoptotic bodies. An alternative pathway of apoptosis may be initiated by injury of phospholipids of the cell membrane, which may induce the sphingomyelin−ceramide signaling system of cell death.[48] A third possible apoptotic pathway is the one in which the activation of Fas receptor by Fas ligand (FasL) induces the formation of an apoptosome complex between Fas-associated death domain (FADD) and procaspase-8 that subsequently activates caspase-8. Then caspase-8 activates the caspase-3−6−7 system that finally cleaves key substrates, and the cell is digested through apoptosis. Caspase-8 may also activate the proapoptotic protein Bid that triggers apoptotic cell death through the mitochondrial pathway.[49]

An outstanding contribution to the study of the biochemical mechanisms of cell death was the discovery, by the end of the 1990s, that intracellular ATP levels dictate whether antitumor drugs, including cisplatin as well as other chemical and physical agents, induce cell death by necrosis or apoptosis and that both processes of cell death are linked.[50−52] Figure 3 shows in discontinuous arrows the currently known interconnections between apoptotic and necrotic pathways. The cleavage of PARP by caspase-3, -6, or -7 switches the cell death mechanism form necrosis to apoptosis. Thus, by inactivating PARP, caspase-3, -6, or -7 relieves necrotic-mediated cell death by virtue of preventing the depletion in NAD^+ and ATP. Caspase blocking by inhibitors of apoptosis (IAPs) plus continued activity by PARP and ATP decrease, by the inhibition of electron transport in broken mitochondria, lead the cell to necrosis because of continuation of PARP-induced ATP depletion.[53] However, in some cases failure to cleave PARP can also lead to apoptosis since depletion of NAD^+/ATP may increase the activity of MPT, thereby promoting ROS, BAX, and Ca^{2+}.[54,55] Altogether, the above-mentioned data indicate that there is a functional cooperativity between apoptotic and necrotic cell death pathways. This hypothesis would explain some unusual observations, indicating that cells might also die as a result of an unfinished apoptotic program. For instance, it has been reported that cisplatin-induced cell death in the L1210 leukemic cell line might be the consequence of a defective apoptotic program that lacks some morphological and biochemical properties attributed to "classic" apoptosis.[56]

3. Molecular Bases of Cisplatin Resistance

The occurrence of resistance is a common drawback of cancer chemotherapy, and *cis*-DDP is no exception.[6] Moreover, the patterns of cisplatin resistance vary considerably between tumor types. Some tumors, such as colorectal cancer and nonsmall cell lung cancer (NSCLC), are intrinsically resistant to *cis*-DDP chemotherapy.[57,58] Other tumor types, such as head and neck cancer, testicular cancer, ovarian cancer, and small cell lung cancer (SCLC), are predominantly sensitive to *cis*-DDP treatment. However, most of these sensitive tumors develop acquired resistance after the initial treatment.[6] In most preclinical models of cisplatin resistance (either acquired or intrinsic), multiple mechanisms appear to operate. Thus, cancer cells that are resistant to *cis*-DDP often exhibit several resistance mechanisms acting simultaneously. The molecular mechanisms of resistance against *cis*-DDP can be divided into two main groups: mechanisms that prevent *cis*-DDP reaching DNA as its main therapeutic target and mechanisms that block the induction of cell death (apoptosis or necrosis) after the formation of the cisplatin−DNA adduct.[6,59] This is schematically outlined in Figure 4.

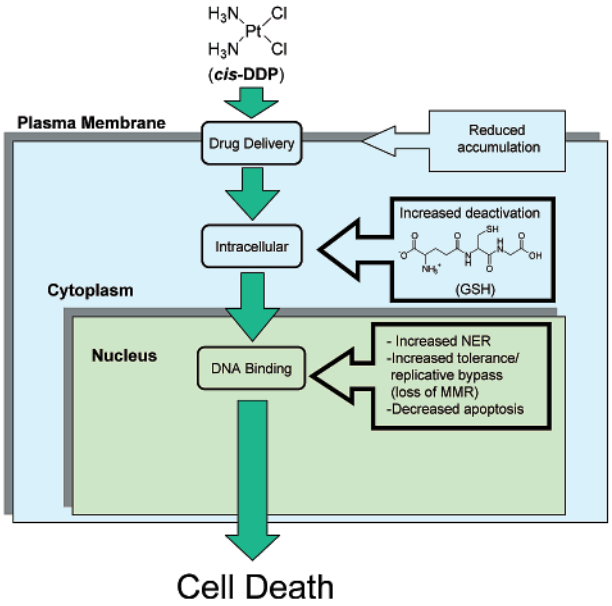


Figure 4. Schematic drawing of the major biochemical mechanisms of resistance to cisplatin. Resistance mechanisms may operate prior to or after binding of *cis*-DDP to DNA. MMR = mismatch repair; NER = nucleotide excision repair; GSH = glutathione.

3.1. Reduced Platinum Accumulation

Decreased uptake/increased efflux of *cis*-DDP leads to lower intracellular concentrations of drug.[60] Most in vitro models of acquired resistance to cisplatin exhibit a decrease in platinum accumulation between 2- and 4-fold.[8] It is generally accepted that reduced platinum accumulation is due to reduce drug uptake rather than to increased drug efflux because the main multidrug resistance efflux pump, P-glycoprotein (Pgp), is not usually overexpressed in cisplatin-resistant tumors.[61] At present, it is known that Pgp is a complex multispanning membrane protein that belongs to the ABC (ATP-binding cassette) transporters. ABC utilizes ATP hydrolysis as fuel to export *cis*-DDP and other antineoplastics against a drug concentration gradient and has been biochemical and pharmacologically characterized.[62,63] However, new multidrug resistance transporters are being characterized, as is the case of the multidrug resistance-associated protein group (MRP), which currently has seven members and also belongs to the ABC family of proteins. It has been recently found that MRP proteins preferably transport drugs (e.g., methotrexate, arsenite, or *cis*-DDP) outside the cell by conjugation with sulfate, glucuronate, or GSH. In fact, the MRP1 and MRP2 proteins confer resistance to *cis*-DDP probably by transporting the drug in complexes with GSH.[64] MRP2 pump is also known as canalicular MRP (cMRP) or canalicular multispecific organic anion transporter (cMOAT). Although the biochemical mechanisms by which *cis*-DDP enters the cells are not fully understood, it appears that passive diffusion is the main way of *cis*-DDP uptake. However, some facilitated or active transport mechanisms may contribute to cisplatin intracellular accumulation.[65] In fact, although *cis*-DDP uptake is not saturable or inhibited by structural analogues, a certain degree of uptake is, at least, energy-dependent and can be modulated by pharmacological agents such as Na^+/K^+-ATPase inhibitors and membrane-interactive drugs.[61,65]

3.2. Cisplatin Inactivation by Thiol-Containing Biomolecules

A more established biochemical mechanism of resistance to cisplatin is the intracellular inactivation of the *cis*-Pt(II) center prior to binding to DNA by coordination to S-donor cysteine residues of the cytoplasmic tripeptide glutathione (see glutathione structure in Figure 4) or metallothioneins (a class of low-molecular-weight proteins).[9] Glutathione (GSH) is a tripeptide of glutamate (Glu), cysteine (Cys), and glycine (Gly) that contains an unusual γ-peptide bond between glutamate and cysteine (γ-GluCysGly). Such a bond prevents GSH from being hydrolyzed by most peptidases. Intracellularly, GSH is kept in its thiol form by glutathione disulfide reductase, a NADPH-dependent enzyme. GSH reacts with cisplatin and other electrophilic compounds to form deactivated conjugates that are readily excreted by a GS-conjugated export pump. This reaction may occur spontaneously or with the help of the glutathione S-transferase enzyme (GST).[66] The removal of platinum by GSH depletes intracellular GSH levels. GSH depletion is known to sensitize cells to many cytotoxic agents including cisplatin through activation of sphingomyelinase (SMase), which increases ceramide levels leading to SMase-induced apoptosis.[67] On the other hand, high intracellular concentrations of GSH (up to 10 mM) often correlate with *cis*-DDP resistance. In fact, prominent GS−Pt−SG complexes (binding ratio of 1 mole of platinum per 2 mol of glutathione) have been found in tumor cells.[8] On the other hand, increased levels of metallothioneins have been also found in some cell lines with acquired

resistance to *cis*-DDP.[6,8] Mammalian metallothionein (MT) is a small protein of 62 amino acids which contains 20 cysteine residues. Mammalian MT has been involved in intracellular detoxification of heavy metal ions such as Cd^{2+} and Zn^{2+}. Cisplatin binds to MT, with a stoichiometry of 10 Pt atoms per MT molecule and with a binding constant rate which is significantly higher than that for GSH. When *cis*-DDP binds to MT, it loses its NH_3 ligands and displaces from MT heavy-metal cations (e.g., Zn^{2+}) according to the reaction

$$(Zn^{2+})_7-MT + 10(NH_3)_2Pt^{2+} \rightarrow (Pt^{2+})_{10}-MT + 20NH_3 + 7Zn^{2+}$$

It is not yet clear whether MT plays a role in *cis*-DDP resistance. However, it has been recently reported that transfection of the human metallothionein MT-II_A cDNA into cells conferred over 4-fold resistance to cisplatin.[68]

3.3. The Role of DNA Adduct Structure and Conformation in Cisplatin Resistance

It is known that even high levels of DNA platination may not always induce cell death.[6] Several proteins have been described that recognize and bind to cisplatin−DNA adducts. These proteins are called "damage-recognition proteins" and include, among others, the XPA−RPA complex, nonhistone chromatin high mobility group HMG1 and HMG2, histone H1, the TATA-box binding protein TBP, and HMSH2.[12] Damage-recognition proteins may either assist in the repair of DNA lesions provoked by *cis*-DDP or, conversely, shield damage from repair proteins.[69]

An important biochemical mechanism of resistance that occurs after platinum binding is the repair of DNA damage. Nucleotide excision repair (NER) appears to be a major mechanism of cisplatin-resistance. Increased NER in cisplatin-resistant cell lines has been shown to occur both for intrastrand as well as interstrand cisplatin−DNA adducts.[2] For the 1,2-intrastrand adducts of *cis*-DDP, the NER system is of particular importance.[70] NER is an ATP-dependent multiprotein complex that recognizes the kink induced on DNA by 1,2-intrastrand cross-links and subsequently excises the segment of the DNA that includes the kink, as a 27−29-base-pair oligonucleotide. The gap that remains is then filled by DNA polymerase.[71] Increased removal by NER of platinum−DNA adducts from the genome of resistant cell lines relative to sensitive parent cell lines has been consistently observed in several models. The increased NER activity in cisplatin-resistant cell lines appears to be most strongly associated with increased levels of expression of ERCC1 and XPA proteins.[12,72] Conversely, defective NER has been found in cell lines with hypersensitivity to *cis*-DDP. In fact, it has been recently reported that the testis specific protein tsHMG, which belongs to the family of HMG domain proteins, might bind to 1,2-d(GpG) intrastrand cross-links blocking DNA repair by NER.[73]

As mentioned above, HMG-box proteins, including HMG1 and HMG2, bind selectively to DNA modified by *cis*-DDP but not to that modified by biologically inactive *trans*-DDP.[74] In contrast to NER proteins, HMG1 is able to inhibit the repair of the major 1,2-d(GpG) intrastrand cisplatin−DNA adduct by human excision nuclease in vitro.[75] Several mechanisms have been proposed to explain how HMG domain proteins might modulate the sensitivity of cells to *cis*-DDP. Two of them seem to be the most feasible ones. The "repair shielding model" postulates that HMG proteins could protect cisplatin−DNA adducts from recognition by DNA repair enzymes.[76] The second one, the so-called "hijacking model", establishes that HMG proteins such as SSRP1 could modulate cell cycle events after DNA damage and trigger cell death. Thus, this latter model postulates that the recognition by HMG cellular factors of cisplatin−DNA lesions would deviate them from their natural binding sites resulting in inhibition of vital cellular functions.[77]

The post-binding mechanism described as "increased tolerance" is probably one of the most general biochemical mechanisms of resistance encountered in cancer chemotherapy of DNA-binding drugs.[78] Post-replication repair is defined as the replication of damaged DNA without the introduction of gaps into the DNA and/or the repair of those discontinuities following replication.[78] Since the presence of gaps or discontinuities in replicated DNA can be lethal, post-replication repair is a major mechanism of DNA damage tolerance. In human cells, post-replication appears to occur primarily during replication so that it is often referred as a replicative bypass. Enhanced post-replicative bypass, the ability of the replication complex to synthesize DNA downstream a cisplatin-induced lesion, has been found in some cisplatin-resistant cells.[79] The biochemical bases of increased tolerance to damaged DNA are still unclear. However, increased post-replicative bypass of cisplatin−DNA adducts has been observed in cell lines with defects on a second DNA repair process named mismatch repair (MMR).[80] The mismatch repair system involves at least five proteins (MLH1, MSH2, MSH3, MSH6, and PMS2) and functions as an ATP-dependent repair process that corrects misincorporated nucleotides.[81] The human MSH2 protein (hMSH2) recognizes 1,2-d(GpG) intrastrand cross-links of *cis*-DDP on DNA.[82] 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−DNA adducts.[8] In cisplatin-sensitive MMR-proficient cells, binding of hMutSα or hMutLα MMR complex to cisplatin−DNA adducts is thought to result in a continuous futile cycle of repair on the opposing DNA strand, ultimately leading to cell death (see Figure 5).[83] It has been recently reported that the registered drug oxaliplatin, which contains the bulky, nonpolar 1,2-diaminocyclohexane (DACH) ligand in place of the ammine ligands of *cis*-DDP (see Figure 1), is able to circumvent cisplatin-resistance in MMR-deficient tumor cell lines.[84] In vitro, MutS binding assays have revealed that puri-

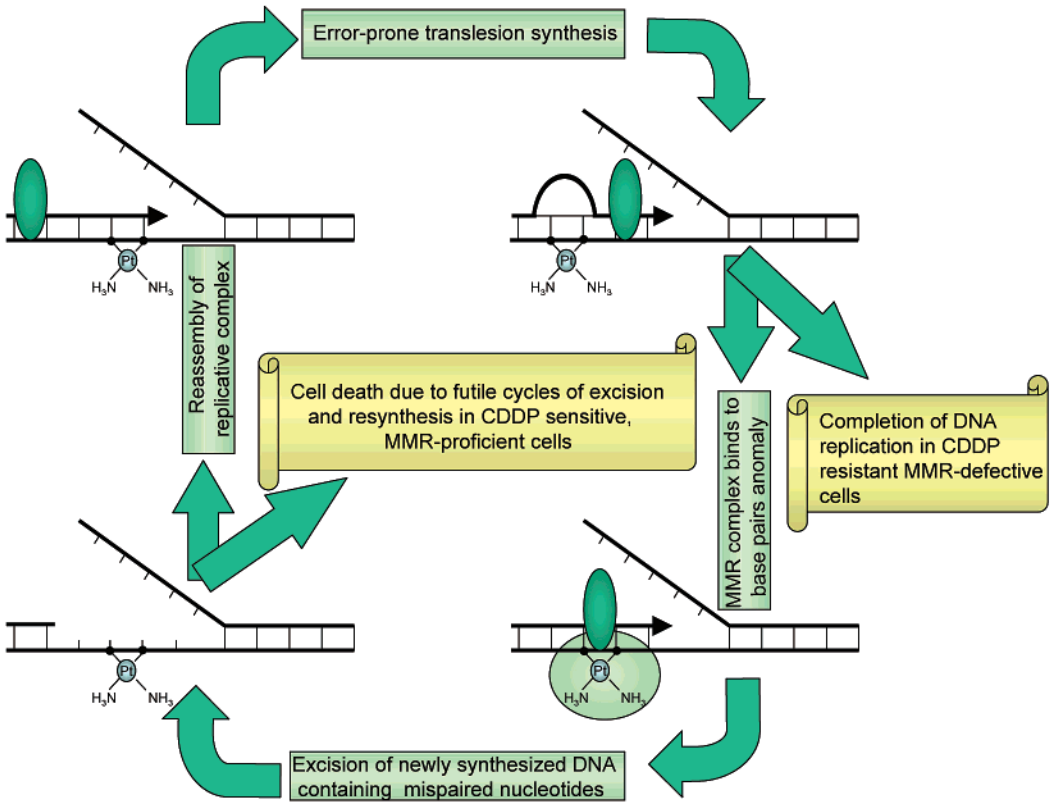


Figure 5. Proposed model for the contribution of mismatch-repair (MMR) activity to *cis*-DDP (CDDP) cytotoxicity. DNA replication downstream the cisplatin 1,2-d(GpG) intrastrand cross-link results in imperfect base pairing. This alteration is recognized by the hMutLα/hMutSα MMR complex. Attempted MMR fails because it is directed at the daughter DNA strand. So the newly synthesized DNA strand is removed, and the intrastrand cross-link on the parental DNA strand remains unexcised. The continued action of these futile replication/repair cycles results in the formation of gaps or strand breaks. An inability to initiate mismatch correction results in cisplatin resistance because these futile repair attempts would be avoided.

fied MutS binds to cisplatin-modified DNA with 2-fold greater affinity than DNA modified with oxaliplatin.[85] So it is likely that structural differences in the conformation of DNA adducts formed by oxaliplatin relative to its analogue *cis*-DDP may be important in the induction of cell killing effects by different biochemical mechanisms. These recent findings support the development of platinum drugs based on their coordination chemistry to combat drug resistance in tumors.[86]

3.4. Failure of Apoptotic Pathways

To trigger apoptosis it is believed that cellular damage has to pass a certain threshold level.[2] However, damaged genes are common in cancer cells in which proteins involved in apoptotic pathways often malfunction. This can make certain types of cancer rather insensitive to *cis*-DDP damage.[87] Upstream factors involved in the cellular response to the damaged DNA mediate the induction of a network that transmits both pro- and antiapoptotic signals. So, any interference that induces antiapoptotic signal transduction or abrogates proapoptotic pathways, including transcriptional and translational responses, is a potential mechanism of *cis*-DDP resistance.

It is known that p53 protein plays a central role in chemotherapy-induced apoptosis. The tumor suppressor gene *p53* facilitates DNA repair before DNA replication. *p53*, considered to be "guardian of the genome", functions as a transcription factor regulating a host of other genes that lead to cell cycle arrest or induction of cell death.[88] In fact, p53 is a strong transcriptional activator of the gene encoding $p21^{WAF1/CIP1}$, a protein that mediates cell cycle arrest and that may also protect cells from apoptosis.[89] It has been recently found that p53 may be involved in the development of *cis*-DDP resistance through the regulation of several genes involved in drug resistance and apoptosis (e.g, mismatch repair, bcl-2, high mobility group proteins, DNA polymerases α and β, PCNA, and insulin-like growth factors).[78] However, a clear relationship between p53 cellular status and cisplatin-induced cytotoxicity has not been found yet. In fact, the cell type and the cellular context exert a strong influence in p53-mediated responses to *cis*-DDP damage of DNA.[43,90]

The *bcl2* family of genes encodes a group of proapoptotic (e.g., Bax, Bak, Bad) and antiapoptotic (e.g., Bcl-2, Bcl-X_L) proteins, which form homo- and heterodimers with one another. It is currently thought that the relative level of pro- and antiapoptotic proteins may function as a cell survival/cell death rheostat to influence sensitivity and resistance to cisplatin-induced apoptosis. So high levels of Bcl-2 may induce resistance to *cis*-DDP through inhibition of apoptosis.[91] On the other hand, Bcl-2 and p53 are often overexpressed in resistant ovarian cancer cell lines and it has been proposed that Bcl-2 might act

upstream of the p53 pathway.[43] However, it has also been found that high levels of Bcl-2 increase sensitivity to *cis*-DDP in human ovarian cancer cells.[92]

Protein kinase A (PKA) may confer sensitivity to *cis*-DDP through apoptosis induction.[93] Protein kinase A, also called cyclic AMP-dependent protein kinase, participates in glycogen breakdown to glucose-1-phosphate (glycogenolysis). PKA is a tetramer having two catalytic subunits, C, and two regulatory subunits, R. The tetramer, R_2C_2, is catalytically inactive, and binding of cyclic AMP (cAMP) to the R subunits causes the tetramer to dissociate, yielding the catalytically active monomer C.[94] Besides, PDE (phosphodiesterase) negatively regulates PKA through a decrease in the intracellular concentrations of cAMP. Decreased cAMP levels may produce resistance to *cis*-DDP through suppression of Bcl-2 expression and inhibition of apoptosis.[95]

Sphingosine-1-phosphate phosphatase (S1PP) is an enzyme that produces sphingosine by cleavage of the ester-phosphate bond of sphingosine-1-phosphate (S1P). The impairment of S1PP activity shifts the balance between ceramide, which as mentioned above induces apoptosis, and S1P, which promotes cell survival.[96] Inhibition of S1PP will lead to accumulation of S1P, thereby protecting cells form apoptosis. However, S1P can also induce cell death in certain cells. So the modulation of the ceramide-sphingosine-sphingosine-1-phosphate rheostat may be an important factor with relation to potential resistance to *cis*-DDP.[97]

In somatic cells, the ends of chromosomes (the telomeres) shorten with each cell division. However, in tumor cells, telomere length is maintained, mainly through activation of the reverse transcriptase enzyme, telomerase.[98] Telomeres are tandemly repeated DNA sequences, comprising a G-rich strand and a complementary C-rich strand, located at the end of the chromosomes. Telomerase is a ribonucleoprotein that uses its RNA component as template to synthesize the 5′-d(TTAGGG)-3′ repeats at the ends of the chromosomes. Thus, to maintain telomere length, malignant cells have to replicate the telomeric motif several times, yielding a $d(TTAGGG)_n$ telomeric sequence.[69] It has been reported that HeLa cells sensitive to low doses of *cis*-DDP may die through apoptosis as a consequence of cisplatin binding to telomeres and subsequent telomere loss.[99] Therefore, a putative resistance mechanism to cisplatin might be related with hyperactivation of telomerase and inability of the tumor cell to engage apoptosis.[100]

4. Biochemical Modulation of Cisplatin Chemosensitivity

Treatment of cisplatin-resistant tumors is a major drawback which may, at least, be partially addressed by using biochemical modulation strategies directed to the enhancement of the activity of *cis*-DDP through manipulation of resistance pathways by pharmacological agents. Table 1 summarizes the resistance mechanisms to *cis*-DDP which have been so far manipulated with biochemical modulators. In addition, Figure 6 shows the structure of several selected biochemical modulators of *cis*-DDP resistance.

Table 1. Selected Biochemical Modulators of Cisplatin Resistance Pathways

biochemical mechanism of resistance	modulator
platinum accumulation	dipyridamole
	amphotericin B
	cyclosporin
platinum detoxification by glutathione	L-buthionine sulfoximine
	diazenes
	ethacrynic acid
repair of Pt−DNA adducts	
	aphidicolin
	gemcitabine
	azidothymidine
	cytarabine
	dideoxythymidine
	deoxyazacytidine
	hydroxiurea
	trifluoperazine
	camptothecin
	nalidixic acid
	novobiocin
	doxorubicin
	etoposide
	estrogen
	progesterone
cell death pathways (ATP-depleting agents)	6-aminonicotinamide
	6-methylmercaptopurine riboside
	N-(phosphonacetyl)-L-aspartic acid

4.1. Cisplatin Accumulation

Decreased accumulation of *cis*-DDP in the tumor cell is one of the most important mechanisms of resistance to the drug both in preclinical and clinical settings.[5,6] As previously mentioned, *cis*-DDP accumulation depends on two factors: drug uptake and drug efflux. The most promising drugs in modulating cisplatin uptake and/or efflux are dipyridamole, amphotericin B, and cyclosporine A.

Dipyridamole (Dpm) is a pyrimido-pyrimidine derivative that decreases cisplatin efflux and increases drug uptake.[101] The mechanism by which Dpm decreases cisplatin efflux is not yet fully understood. In animal cells a single protein, the nucleoside transporter, appears to be responsible for uptake of a wide variety of nucleosides including adenosine. Functioning of this protein is inhibited by Dpm, which blocks the uptake of adenosine.[102] It is thought that dypiridamole may exert an indirect effect in *cis*-DDP accumulation by decreasing ATP-dependent drug efflux mechanisms associated to multidrug resistant transporters.[103] In addition, Dpm may enhance *cis*-DDP accumulation by increasing cell membrane permeability. It has been reported that Dpm increases the cytotoxic activity of cisplatin in human colon carcinoma cells as well as in human ovarian cancer cells.[103,104] Moreover, a combination of 5-fluorouracil (5FU), *cis*-DDP, and Dpm has proven to be highly effective in patients with advanced gastric cancer. 5FU is a clinically used antineoplastic drug, which inhibits pyrimidine biosynthesis.[105] Combinations of 5FU plus *cis*-DDP have been given clinically to treat solid tumors including, head and neck cancers and gastrointestinal malignances.[101]

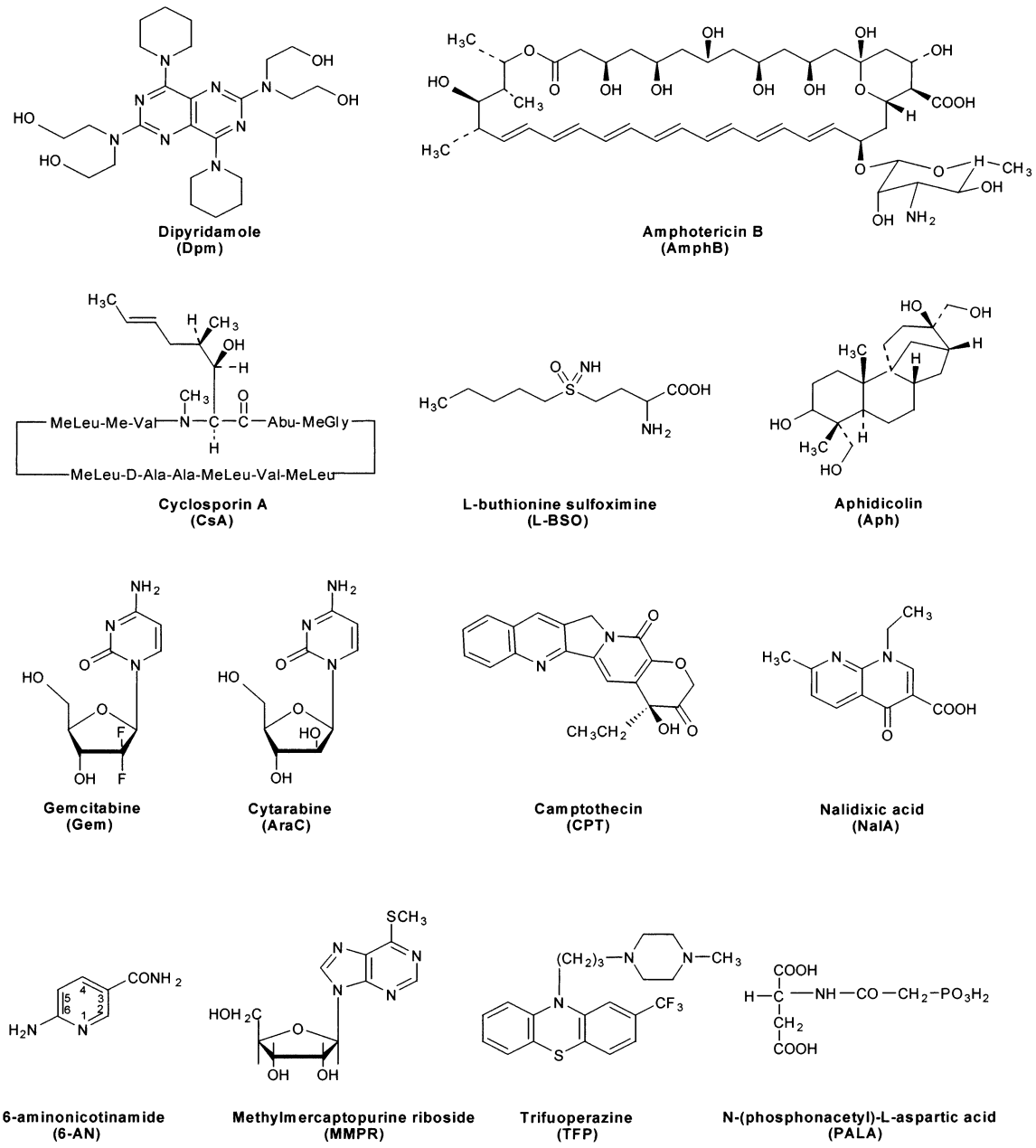


Figure 6. Structures of selected biochemical modulators of *cis*-DDP resistance. In some structures the aromatic rings are numbered.

Amphotericin B (AmphB) is a polyene antifungal antibiotic which is known to increase *cis*-DDP cytotoxicity in some preclinical models.[106] AmphB molecule (see Figure 6) contains lactone and alcohol functions, conjugated double bonds, and a mycosamine sugar ring. Conjugated double bonds and OH groups allow AmphB to interact through van der Waals forces and hydrogen bonding with sterols of the cell membrane of animal cells. The result is the formation of membrane pores which provoke K^+ efflux.[107] The efflux and influx of K^+ ions plays an important role in the induction of apoptosis.[108] It has been recently reported that the combination of *cis*-DDP or carboplatin with amphB plus the $Na^+-K^+/2Cl^-$ cotransport blocker bumetadine potentiates apoptosis in pulmonary mesothelioma P31 cells.[109]

Cyclosporine A (CsA), a nonpolar cyclic oligopeptide of 11 residues containing some rare amino acids, has been traditionally used as an immunosupressor. The combination of *cis*-DDP plus CsA has been shown to have a promising degree of activity in patients with recurrent and cisplatin-resistant ovarian cancers.[110] CsA improves *cis*-DDP accumulation in culture cells by inhibiting several ATP-dependent drug efflux pumps. Thus, CsA reverses the drug resistance conferred by overexpression of both P-glycoprotein (PgP) and the canalicular multispecific organic anion transporter (cMOAT).[111,112] CsA has also been shown to decrease the resistance of cancer cells to cisplatin-modulating signal transduction pathways, which include suppression of cisplatin-induced *c-fos* oncogene expression.[113]

4.2. Platinum Detoxification by Glutathione

Glutathione (GSH) is an attractive target for biochemical modulation because it potentially affects *cis*-DDP sensitivity through several mechanisms. GSH can bind to cisplatin in the cytoplasm or to

platinum−DNA monofunctional adducts in the nucleus preventing the formation of potentially cytotoxic cross-links.[114,115] GSH−platinum complexes are actively transported out of the cells (ATP-dependent efflux) contributing to reduced drug accumulation.[116,117] In addition, GSH may directly or indirectly participate in DNA repair because depletion of GSH by several drugs inhibits DNA repair.[118] Moreover, active cysteine residues of HMG1 and HMG2 damage recognition proteins must be in a reduced form to be able to recognize cisplatin−DNA intrastrand cross-links.[119] GSH may also modulate the expression of transcription factors that potentially affect DNA repair and apoptosis, such as c-*fos* and c-*jun*.[6,120] GSH is synthesized intracellularly in a two-step pathway that is ATP-dependent (reactions 1 and 2):

$$\text{L-Glu} + \text{L-Cys} + \text{ATP} \leftrightarrow \text{L-}\gamma\text{-Glu-L-Cys} + \text{ADP} + \text{HPO}_4^{2-} \quad (1)$$

$$\text{L-}\gamma\text{-Glu-L-Cys} + \text{Gly} + \text{ATP} \leftrightarrow \text{GSH} + \text{ADP} + \text{HPO}_4^{2-} \quad (2)$$

The rate-limiting step in the synthesis of GSH is reaction 1, in which an amide bond between the NH_2 group of cysteine (Cys) and the γ-COOH group of glutamate (Glu) is formed. The enzyme γ-glutamylcysteine synthetase (γ-GCS) catalyzes this first reaction and can be inhibited by L-*S*,*R*-buthionine sulfoximine (L-BSO). Reaction 2 uses the enzyme glutathione synthetase (GS) to complete the tripeptide synthesis.[121] L-BSO acts as transition state analogue so that γ-GCS catalyzes the Mg^{2+}/ATP-dependent phosphorilation of L-BSO to yield L-BSO phosphate, a tightly bound enzyme inhibitor. Enzyme inhibition follows pseudo-first-order kinetics, is noncovalent, and is apparently irreversible in the presence of Mg^{2+}/ATP.[122] It appears that enzyme inhibition is stereospecific because only the L-*S*,*R* enantiomer of BSO actually inhibits γ-GCS.[123] Depletion of GSH with L-BSO enhances the cytotoxicity of cisplatin-resistant tumors in several in vitro and in vivo preclinical models.[124,125] Moreover, it has been reported that L-BSO may be useful as a modulator of *cis*-DDP cytotoxicity rhythms in mice bearing PO3 pancreatic adenocarcinoma.[126] The limited availability of L-BSO for clinical use has so far impeded relevant attempts to define its clinical utility as a modulator of *cis*-DDP resistance. However, L-BSO has proved its efficacy as an enhancer of the antitumor activity of the alkylating agent melphalan in Phase I and II clinical trials.[127] Interestingly, alternative inhibitors of the glutathione system such as diazenes and ethacrynic acid are currently under preclinical investigation.[101,128]

4.3. DNA Repair and Processing

The possible clinical role of increased repair/tolerance of cisplatin−DNA adducts, as a major mechanism of *cis*-DDP resistance, has led to attempts to enhance the therapeutic effect of *cis*-DDP by combination with either DNA repair inhibitors or drugs involved in DNA processing and topology.[129]

Eukaryotic cells contain five distinct DNA polymerases: α, β, γ, δ, and ϵ. These enzymes are distinguished from each other by their intracellular locations, kinetic properties, and responses to inhibitors.[130] Polymerases α, β, and ϵ are located in the cell nucleus and all play an essential role in DNA replication. Moreover, polymerases α, β, and ϵ are sensitive to inhibition to the diterpene antibiotic aphidicolin (Aph).[130] Polymerase α is responsible for the replication of the DNA strand called "lagging strand" that is synthesized in the opposite direction to the movement of the replication fork. The lagging strand is synthesized as stretches of DNA known as Okazaki's fragments, which are subsequently joined by DNA ligase.[131] The administration of aphidicolin glycinate, a water-soluble form of the steroid Aph, prior to administration of *cis*-DDP, markedly increased the survival rate of athymic nude mice with intraperitoneally implanted cisplatin-resistant OVCAR-3 ovarian cancer cells, as compared to either agent used alone.[132] It has been also reported that the nucleoside analogue gemcitabine (Gem) is able to enhance the antitumor efficacy of cisplatin. Gem is an inhibitor of DNA polymerase-mediated chain elongation and exonuclease repair.[133] Other nucleoside analogues are involved in inhibition of DNA replication by stopping chain elongation in the "leading strand" (DNA strand which is in the direction of the movement of the replication fork). These drugs may also block nucleotide gap filling in the lagging strand. On the other hand, nucleoside analogues such as azidothymidine (AZT), arabinosylcytosine (cytarabine, AraC), and dideoxythymidine (ddT) are able to increase the cytotoxicity of *cis*-DDP in tumor cell lines resistant to the drug.[6,129] The nucleoside analogues, lacking a 3′ hydroxyl terminus, function as blockers of DNA replication once they are converted within the cell to the corresponding triphosphate nucleotide by its incorporation into DNA.[134] Increased *cis*-DDP binding to DNA, through an alteration of DNA topology, has been proposed as a biochemical mechanism for the synergistic interaction of the DNA methyltransferase inhibitor 2′-deoxy-5-azacytidine (DAC) with *cis*-DDP. The enhanced binding of cisplatin to DAC-modified DNA was found to be independent of DNA hypomethylation because methylated plasmid DNA bound more *cis*-DDP than unmethylated plasmid DNA.[135]

Hydroxyurea ($H_2NCONHOH$) is a DNA repair inhibitor, which acts by blocking ribonucleoside diphosphate reductase (rNDP). This enzyme reduces all four common ribonucleotide diphosphate substrates to the corresponding 2′-deoxyribonucleotides.[136] Mechanistically, the reaction proceeds with retention of configuration at C2′ of the sugar ring, which rules out the displacement of the hydroxyl group by a hydride ion in a S_N2 reaction. rNDP contains catalytic residues on each of its subunits, which are redox-active thiols of cysteine residues and a tyrosine free radical stabilized by an Fe^{3+}−oxygen complex. The SH groups undergo oxidation during the reaction. In addition, it is believed that the tyrosine free radical participates in the reaction because the rNDP inhibitor hydroxyurea reversibly destroys the free radical.[137] Combinations of *cis*-DDP and other platinum analogues with hydroxyurea have been shown to work

synergistically in experimental models of cisplatin resistance.[5]

Calcium channel blockers such as verapamil, nifedipine, and the phenotiazine derivative trifluoperazine (TFP) may increase the cytotoxic activity of *cis*-DDP in human ovarian cancer cells.[138] TFP is a calmodulin antagonist, which exerts its pharmacological action at several levels, including specific interactions with calmodulin and/or P-glycoprotein, interactions with the tumor cell plasma membrane, and an indirect inhibition of DNA repair.[101,139]

Also, it is known that DNA supercoiling may influence binding of cisplatin to DNA. In fact, the formation of DNA interstrand cross-links by *cis*-DDP is thermodynamically favored in negatively supercoiled DNA owed mainly to the relaxation of supercoils.[140] Conversely, binding of cisplatin to DNA alters DNA supercoiling. For instance, binding of *cis*-DDP to negatively supercoiled plasmid DNA unwinds the DNA superhelix.[141] In addition, it has been reported that *cis*-DDP inhibits DNA gyrase, alters DNA supercoiling, and enhances DNA gyrase gene expression.[142] Topoisomerases (Topo) are enzymes that play an important role in DNA supercoiling and affect specific processes including DNA transcription, DNA duplication, and chromosome segregation. Type I Topoisomerases break and reseal one DNA strand. Type II Topoisomerases catalyze double-strand breakage and rejoining of DNA.[143] Topo I enzymes completely wraps around its DNA substrate so that the DNA−protein contacts involve the DNA sugar−phosphate backbone. Camptothecin (CPT) is a Topo I inhibitor that binds within the DNA−protein contacts on the upstream 5′-side of the scissile phosphodiester bond.[144] Interestingly, the combination of *cis*-DDP with CPT has shown to have a synergic effect in human tumor xenografts and it is currently used in clinical trials.[144,145] DNA gyrase is an *E. coli* enzyme that belongs to the topoisomerase II family and can relax supercoiled DNA, introducing negative superhelical turns. ATP hydrolysis is required during the catalytic cycle of DNA gyrase and most Topo II enzymes. DNA gyrase is a tetramer, with two A and two B subunits. The A subunits bind and cleave DNA, while the B subunits carry out the energy transduction resulting from ATP hydrolysis. The gyrase A subunit is the target for the binding of nalidixic acid (NalA), a quinoline derivative that inhibits DNA replication.[146] Another replication inhibitor, the anthracyclin novobiocin, binds to the B subunit of gyrase and inhibits ATP cleavage.[147] In a human glioblastoma cell line, novobiocin pretreatment caused a 3-fold increase in the sensitivity of cells to *cis*-DDP. This increase in cisplatin cytotoxicity was associated with an enhancement of total genomic interstrand cross-links and a reduction in the rate and extent of cross-link repair.[148] Human Topo II enzymes are located in the cell nucleus and prevent the formation of "knots" in DNA by allowing the passage of an intact segment of the helical DNA through a transient double strand break.[148] There are two Topo II enzymes in human beings, named α and β. Topo IIα is expressed preferentially in the G2/M phase of the cell cycle, while Topo IIβ is expressed constitutively during the cell cycle.[143] Human Topo II inhibitors, which stabilize the Topo II−DNA complex and interfere with DNA rebinding, are called Topo II poisons. Within these inhibitory drugs, anthracyclines, as doxorubicin, and epipodophyllotoxins, as etoposide, are included (see Topo II catalytic cycle and etoposide structure in Figure 7). Doxorubicin (DOX), also known as adriamycin, is a planar aromatic molecule that binds to DNA by intercalation between nucleobase pairs forming π-stacks.[150] Three principal functional components of DOX have been identified: (1) the intercalator (rings B−D), (2) the anchoring function associated with ring A (e.g., C9−OH group), and (3) the amino sugar (see Figure 8). It has been recently shown that under certain redox conditions DOX is capable of forming a covalent adduct with DNA using the daunosamine N3′ atom of the drug and the guanine N2 amino group.[151] The DOX-induced DNA cross-link may be the result of the action of HCOH (formaldehyde) generated from the DOX molecule via the Baeyer−Villiger reaction (see also Figure 8).[152] The combination of DOX and *cis*-DDP has been successfully used in the clinic for the treatment of advanced-stage ovarian cancer as well as metastatic breast cancer.[153] The Topo II inhibitor etoposide, also called VP-16, binds to the complex formed by Topo II and the 5′-cleaved ends of the DNA, thus forming nonrepairable protein-linked DNA double strand breaks.[154] VP-16 also exhibits a synergistic effect with *cis*-DDP. The combination of VP-16 with *cis*-DDP had greater cytotoxic activity against four of five cell lines of cisplatin-resistant head and neck cancer, when compared to any single drug used alone.[155] Moreover, the combination of *cis*-DDP with VP-16 is widely used for the treatment of patients with primary or recurrent small cell lung cancers.[156]

A novel and promising strategy for biochemical modulation of cisplatin resistance is the inhibition of DNA repair through drugs which induce an increase in the levels of HMG domain proteins in tumor cells. For instance, it has been reported that treatment of MCF-7 breast cancer cells, having the steroid hormone receptors, with the appropriate hormone, estrogen, and/or progesterone, significantly increases the potency of cisplatin and its analogue carboplatin by causing the overexpression of HMG1.[157]

4.4. Cell Death Pathways

As mentioned already, *cis*-DDP provokes DNA damage which initiates the cell death pathways of apoptosis and necrosis.[158] In certain cases of cisplatin resistance, apoptotic cell death pathways may be inhibited due to a drastic reduction of the bioenergetic cellular index (BEC index).[159] Thus, tumor cells with a low BEC index, as a result of a low mitochondrial content and/or activity, would become more resistant to programmed cell death. On the other hand, in highly cisplatin-resistant sublines, necrotic cell death may be blocked due to the inactivation of poly(ADP−ribose) polymerase (PARP) cleavage of NAD^+.[160] As early as 1956, Otto Warburg reported that an elevated rate of glycolysis is a common feature of most tumors.[161] Glycolytic metabolism is

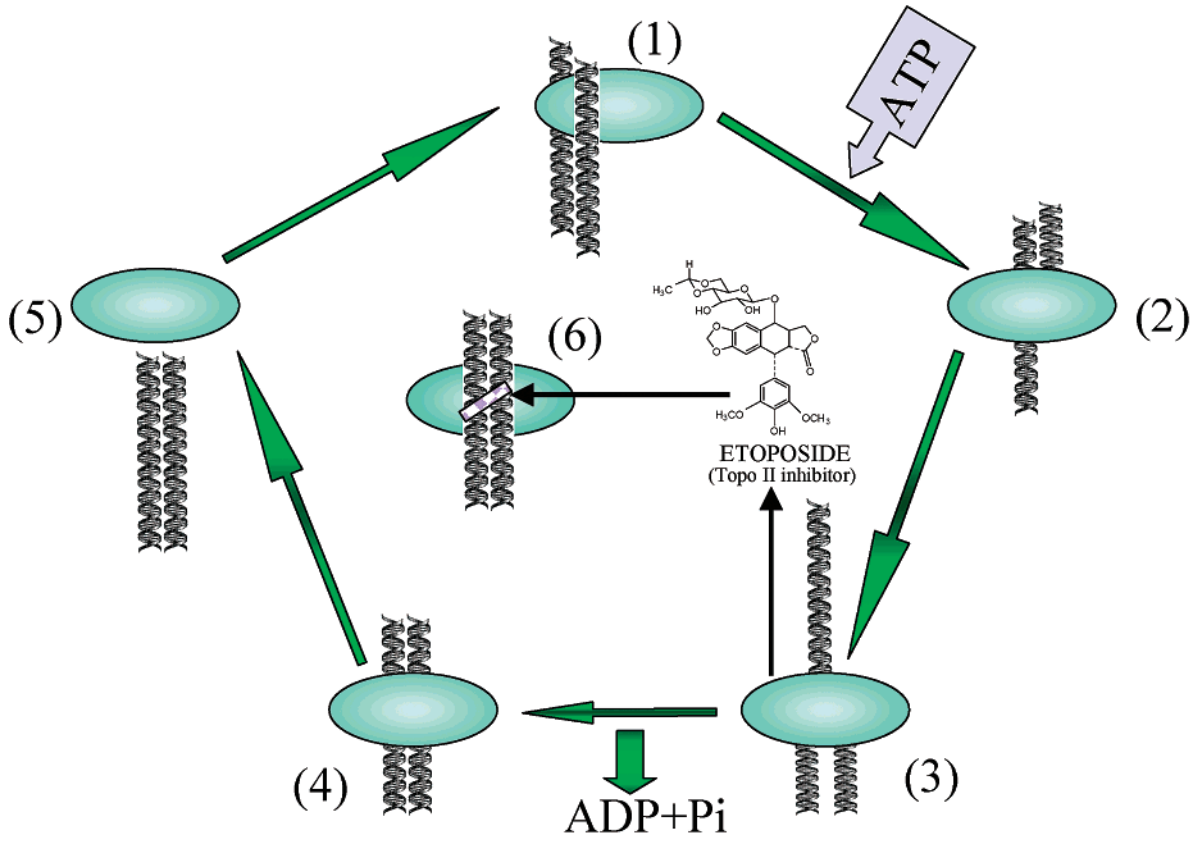


Figure 7. Catalytic cycle of topoisomerase II (Topo II). The catalytic cycle of Topo II consists of the following individual steps. Step 1: Topo II initiates its catalytic cycle by binding to its DNA substrate. Step 2: in the presence of a divalent cation (e.g., Mg^{2+}), the enzyme establishes a prestrand passage DNA cleavage/religation equilibrium. Step 3: Topo II cuts DNA at preferred sequences, established within the binding/recognition sites. Step 4: upon binding of its ATP cofactor, Topo II undergoes a structural reorientation that triggers double-strand DNA passage. Step 5: Topo II dissociates from its DNA substrate and may initiate another round of the catalytic cycle. Step 6: DNA religation may be inhibited by some Topo II-target drugs, such as etoposide, that block by weak interactions (e.g., van der Waals forces, hydrogen bonds, and electrostatic bonds) the dissociation of Topo II from its DNA substrate. Pi = inorganic phosphate.

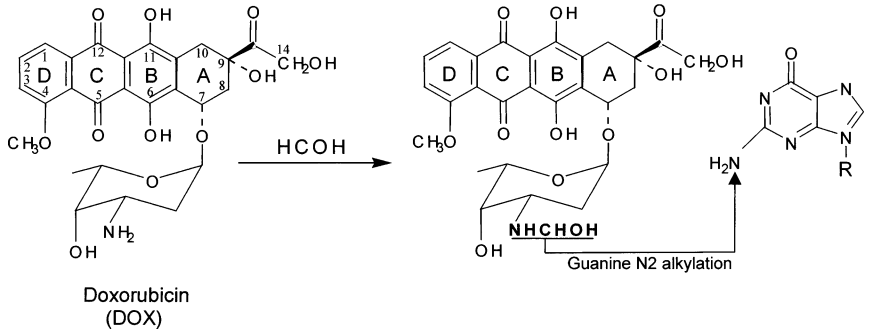


Figure 8. General mechanism of the reaction involving the formation of a covalent bond between the N2 of guanine and doxorubicin through an immonium/imine intermediate (underlined).

important not only for the preservation of ATP generation but also for the maintenance of the hexose monophosphate pathway, a critical generator of reductant power (NADPH) in the cell.[4] Among other functions, NADPH is essential for the maintenance of reduced glutathione (GSH). As mentioned already, GSH is an important intracellular antioxidant and may protect tumor cells against apoptotic cell death through the preservation of protein sulfhydryl groups in a reduced state.[162] Moreover, both ATP and pyridine nucleotide levels in sublethally injured tumor cells are reduced but not to levels sufficiently low to sustain cell viability. So a therapeutic approach based on biochemical modulation to further depress these key nucleotidic metabolites to lethal-inducing levels may provoke the killing of sublethally injured cells through necrotic cell death.[3,163] This therapeutic strategy had its origin in the discovery that extensive drug-induced DNA damage causes DNA strand breaks by failure of the mistmatch repair (MMR) leading to activation of the PARP enzyme.[160] As p53 protein, the PARP enzyme has also been described as a "guardian of the genome integrity". PARP may bind to both single and double strand DNA via Zn^{2+} fingers.[164] In the event of irreparable DNA damage, PARP cleaves the glycolitic coenzyme NAD^+ and transfers single or multiple ADP−ribose moieties (ADPR) to carboxyl groups of nuclear proteins (see Figure 9). The sub-

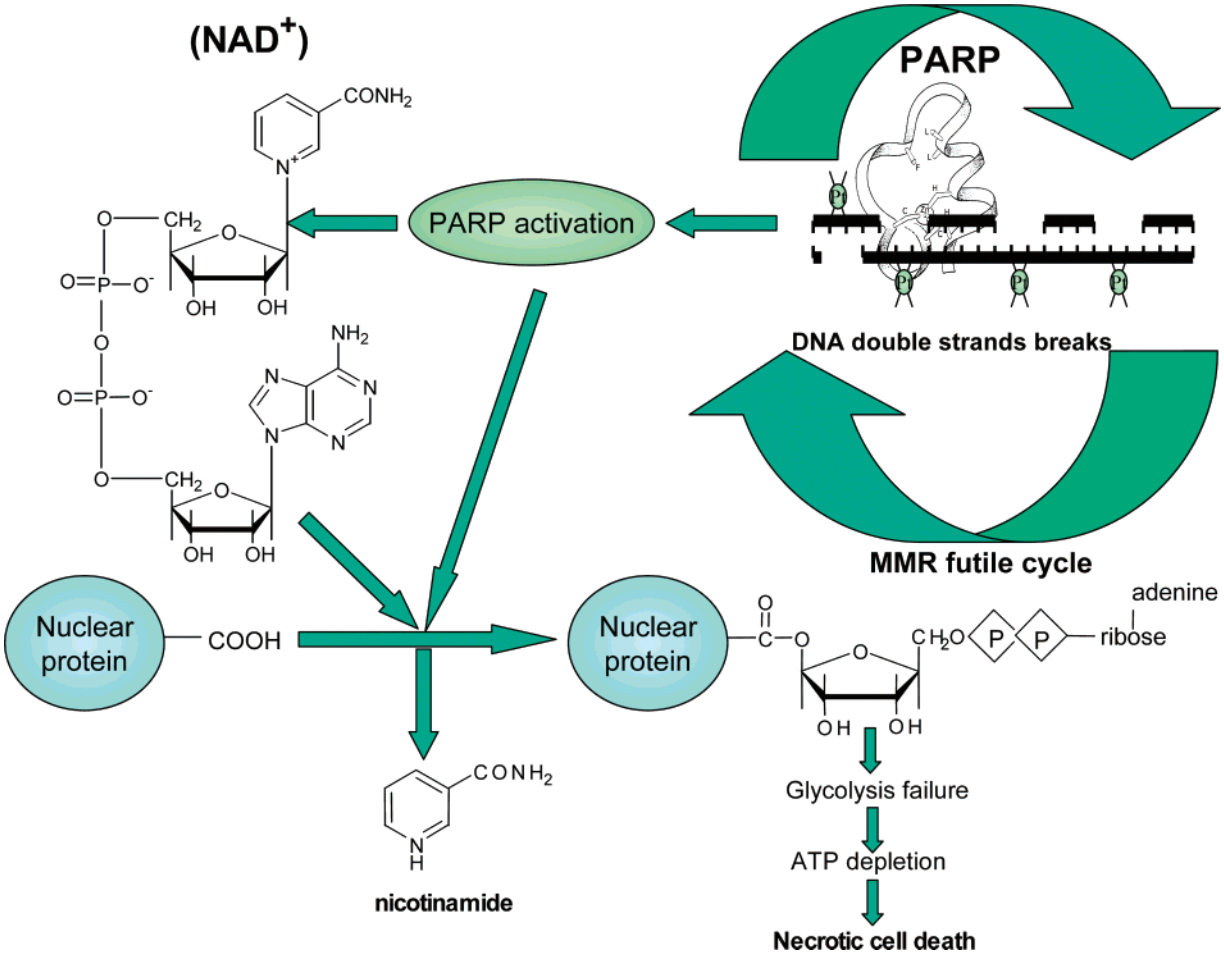
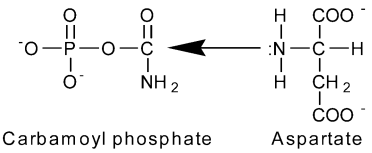
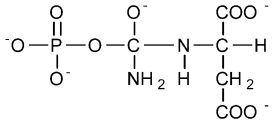


Figure 9. Cleavage of NAD^+ and subsequent transfer of ADP−ribose moieties (ADPR) to carboxyl groups of nuclear proteins through the catalytic activity of poly(ADP−ribose) polymerase (PARP). Extensive DNA damage by *cis*-DDP may induce double strand breaks due, for instance, to mismatch repair (MMR) failure after futile replication/repair cycles. DNA double strand breaks activates PARP, which in turn binds to DNA through a zinc finger (the zinc finger may contain the following: L, leucine; F, phenylalanine; H, histidine; and C, cysteine). PARP also cleaves NAD^+ and catalyzes the incorporation of ADPR units to nuclear proteins ([P] = phosphate group). The PARP reaction involves the binding of the C1′ of ribose to the oxygen of the OH group of the carboxylic function of amino acids of nuclear proteins. NAD^+ depletion inhibits glycolysis with subsequent depletion of ATP levels. If ATP depletion falls to lethal-inducing levels, then, necrotic cell death occurs.

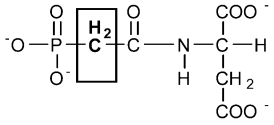
sequent depletion of NAD^+ inhibits glycolytic generation of ATP with consequent ATP depletion. If ATP depletion reaches lethal-inducing levels, then necrotic cell death occurs.[160] There is a likely rationale for the induction of necrotic cell death via PARP activation. A severely damaged cell sustains such a large number of mutations and metabolic alterations that it is seriously impaired in function. Therefore, poly(ADP)-ribosylation may be a system of cell death which operates when the cell is so badly damaged that ATP levels are exhausted and when apoptotic pathways cannot take place. PARP has been very recently identified as a novel target for therapy of important pathologies including cancer, neurologic alterations, and immunological diseases.[164] For instance, it has been reported that a concomitant ATP-depleting strategy, called MAP regime, enhances antitumor drug-induced cell killing in sublethally injured cancer cells through activation of the PARP-associated biochemical mechanism of necrotic cell death.[3] MAP has proven to enhance the cytotoxic activity of several antitumor agents, including doxorubicin, etoposide, paclitaxel, 5-fluorouracil, and cisplatin. The MAP regime is a combination of methylmercaptopurine riboside (MMPR) plus 6-aminonicotinamide (6-AN) plus *N*-(phosphonacetyl)-L-aspartic acid (PALA). 6-AN is a NAD^+ antagonist, which inhibits glycolytic production of ATP.[165,166] MMPR is an inhibitor of de novo purine biosynthesis and, therefore, limits adenine supplies for ATP production.[167] PALA inhibits aspartate transcarbamilase (ATCase) and selectively lowers pyrimidine nucleotide levels in tumors.[168] ATCase is a key enzyme in pyrimidine nucleotide synthesis which catalyzes the formation of carbamoyl aspartate from carbamoyl phosphate and aspartate. PALA acts as an analogue of the bisubstrate transition-state complex formed between carbamoyl phosphate and aspartate within the catalytic center of ATCase (see Figure 10).[169] PALA, due to its high negative charge, binds electrostatically to three arginine (Arg) residues and one lysine (Lys) residue in the ATCase catalytic center. In addition, PALA interacts through hydrogen bonding with other amino acids of the catalytic site. Of particular interest is the hydrogen bond formed between a nitrogen of the imidazol ring of histidine 134 (His_{134}) and the oxygen



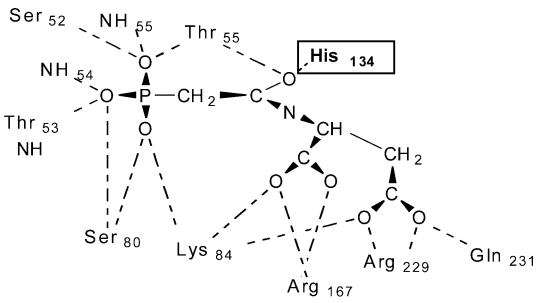
a. **Bound substrates**



b. **Bisubstrate transition-state**



c. **Transition-state analog**



d. **Transition-state inhibitory complex**

Figure 10. Inhibition of aspartate transcarbamylase (ATCase) activity by *N*-(phosphonacetyl)-L-aspartic acid (PALA). (a) Nucleophilic attack of the amine group of aspartate to the carbonylic carbon atom of carbamyl phosphate. (b) Bisubstrate transition state complex. (c) PALA looks like the carbamoyl aspartate bisubstrate complex. The main difference between PALA and carbamoyl aspartate is located around the $-CH_2-$ included in a box. (d) Mode of binding of PALA to the catalytic center of ATC Ase. In this schematic diagram are not indicated all the electrostatic and hydrogen bonds which are formed. In the transition-state inhibitory complex one N atom from the imidazol ring of histidine 134 (His_{134}, within a box) of the ACTase catalytic chain may stabilize, through hydrogen bonding, the negative charge on the oxygen atom of the carbonylic group of PALA.

atom of the carbonyl group of PALA, which stabilizes the negative charge of that carbonyl group. The result is that PALA binds strongly to ATCase with a dissociation constant (K_D) of 10 nM. Moreover, PALA inhibits very efficiently ATCase with and inhibitory constant (K_I) of 10^{-8} M. The MAP regime not only depletes ATP levels but also affects the pyridine nucleotide pool (NAD^+/NADH plus $NADP^+$/NADPH). As mentioned already, intracellular ATP levels may determine whether antitumor drug-induced cell death fate is necrosis or apoptosis.[50,51] It has been proposed that ATP depletion to lethal levels by MAP regime prevents caspase activity to complete anticancer drug-induced apoptosis because caspase-3 may not cleave PARP (see Figure 3).[3] Then ATP depletion is further continued via PARP-induced NAD^+ depletion so that the cell is forced to die by necrosis because there is not enough energy to support apoptosis.[170] Interestingly, the combination of the MAP regime with *cis*-DDP dramatically enhanced the antitumor activity of cisplatin advanced tumor-bearing mice with a variety of cancer types including, breast and colon solid tumors as well as leukemia.[3,171]

5. Concluding Remarks and Future Trends

In most preclinical models of *cis*-DDP resistance (either acquired or intrinsic), multiple biochemical mechanisms appear to work at the same time. Thus, a combination of effects that result in a reduction in the amount of platinum binding to DNA and in an inefficient induction of tumor cell death is very common. Among the many mechanisms of *cis*-DDP resistance described in preclinical models, increased repair/tolerance of platinum−DNA adducts seems to be the most important one in the clinical setting. On the other hand, at levels of cisplatin resistance higher than 40-fold over baseline, increased levels of cellular GSH may play a key role in the resistance to the drug. Decreased cisplatin accumulation is also consistently found both in preclinical and clinical models.[87,172] In addition to "classic" cisplatin resistance mechanisms, several new mechanisms are currently being characterized at the molecular level. In particular, in the preclinical setting, failure to engage apoptosis appears to be another type of broad-spectrum mechanism of cisplatin resistance.[173] However, at present, the clinical relevance of this mechanism is unknown because resistance mechanisms to chemotherapy are not readily studied in cancer patients. Nevertheless, the elucidation of the major in vitro biochemical mechanisms of *cis*-DDP resistance is essential to design strategies to combat the lack of sensitivity to the drug exhibited by some important tumor types, including colorectal and nonsmall cell lung cancers.[160] One of these strategies is the development of platinum compounds capable of circumventing *cis*-DDP resistance, as is the case of oxaliplatin. However, this approach has proven to be a difficult task. In fact, after more than three decades of intensive research, only a very small number of platinum complexes able to circumvent cisplatin-resistance have been discovered.[9] Moreover, only one of these platinum compounds active against cisplatin-resistant tumors, oxaliplatin, is currently registered.[86] Fortunately, the understanding of the major mechanisms of cisplatin resistance has also opened new directions for pharmacological manipulation of cisplatin chemotherapy. So the use of biochemical modulation strategies directed to circumvent cisplatin resistance is a complementary way to the development of novel platinum antitumor drugs. Some biochemical modulators of *cis*-DDP resistance such as the Topo I inhibitor camptothecin and the Topo II inhibitors doxorubicin and etoposide are already used in combination with cisplatin in cancer

patients producing good responses rates.[153] In addition, the modulator of cisplatin accumulation, cyclosporine A, increased the activity of cisplatin in patients with recurrent and platinum-resistant ovarian cancer.[110] Moreover, depletion of GSH levels in tumors by L-BSO administration has proven to work in the clinical setting, although the limited availability of L-BSO has so far precluded its clinical use as a modulator of *cis*-DDP resistance.[127] Of interest is the observation that ethacrynic acid, an inhibitor of glutathione transferase (GST) has shown a synergistic effect with *cis*-DDP in preclinical models being a good candidate for clinical trials.[174] Nevertheless, and despite all these relevant clinical advances, a lot of biochemical modulators of cisplatin resistance still remain in a preclinical phase of development. As noted above, this is mainly due to the great delay that usually exists between the end of the preclinical phase of drug research and the implementation of clinical trials.

In recent years, increasing knowledge of the biochemical mechanisms of drug-induced tumor cell death has opened novel and promising ways for biochemical modulation of the activity of anticancer drugs, particularly in highly resistant tumor cell populations. Of particular interest is the manipulation of tumor cell energy to increase the activity of DNA-damaging antitumor drugs through the induction of necrotic cell death. Thus, the biochemical modulation by MAP regime of *cis*-DDP activity to provoke necrotic cell death in cisplatin-resistant cells merits further research.[3,171] Interestingly, the combination of PALA with cisplatin does not provoke severe toxicity in mice. Moreover, 6-aminonicotinamide (6-AN), as a single agent, has been safely administered to patients with disseminated cancer in Phase I clinical trials.[3] In view of these data, we think that future cancer chemotherapy must be directed to look for adjuvant drugs that affect general biochemical mechanisms that can bypass drug resistance rather than to exclusively search for specific drugs which target particular cellular constituents. Among others, it is the preclinically proven ATP-depleting modulatory concept what warrants and requires appropriate clinical exploration not only when PALA is combined with *cis*-DDP, but also when other ATP-depleting agents are combined with anticancer drugs.

In summary, the data reviewed herein indicate that biochemical modulation of cisplatin mechanisms of resistance offers multiple opportunities for future applications in clinical cancer research. In addition, biochemical modulation may constitute a therapeutic strategy complementary to the discovery of novel platinum complexes with activity in cisplatin-resistant tumors.

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