Coordination Chemistry Reviews 253 (2009) 2070–2081

Contents lists available at ScienceDirect

Coordination Chemistry Reviews

journal homepage: www.elsevier.com/locate/ccr

Review

Mechanistic insight into the cellular uptake and processing of cisplatin 30 years after its approval by FDA

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Contents

ARTICLE INFO

Article history:
Received 21 November 2008
Accepted 27 January 2009
Available online 5 February 2009

Dedicated to the memory of Dr. Lloyd R. Kelland, a great advocate for platinum anticancer drugs.

Keywords:
Platinum-DNA
Antitumor drugs
Cellular uptake
Copper transporter 1
CTR1
Chirality effect

ABSTRACT

When cisplatin was first synthesized, over 150 years ago, inorganic chemistry was in its infancy and no one would have foreseen a role in medicine. Some 120 years later, Rosenberg discovered by serendipity the antitumor activity of this compound but, again, no one would have thought of the existence of specific proteins able to transport platinum across cell membranes or to specifically recognize DNA modified by this drug. However such proteins do exist and, furthermore, are specific for the platinum substrate considered. In this review article the issue of how copper transporter 1 protein (CTR1) can assist the platinum species in entering the cell will be addressed. Platinum binding to double-stranded DNA generates a kinked chelated structure that is recognized by certain proteins in the nucleus, with a direct or indirect consequence on cell viability and induction of apoptosis. Different processing of DNA cross-links formed by species containing enantiomeric diamine carrier ligands (including the carrier ligand of oxaliplatin) will be highlighted.

Abbreviations: ATP, adenosine triphosphate; bp, base pair(s); CDK2, cyclin dependent kinase 2; CTR1, copper transporter 1; DAB, 2,3-diaminobutane; DACH, 1,2-diaminocyclohexane; d(GpG), guanine bases N9-bridged by a deoxyribophosphodiester backbone; DNA, deoxyribonucleic acid; ESI-MS, electrospray mass spectrometry; FDA, Food and Drug Administration; GSK-3, glycogen synthase kinase 3; GST, glutathione-S-transferase; HMG, high-mobility group; HMGB1, protein containing HMG domain; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; NER, nucleotide excision repair; NMR, nuclear magnetic resonance; SOD1, superoxide dismutase 1; TLS, trans-lesion synthesis; TZ6, 2-[6,8-dichloro-2-(1,3-thiazol-2-yl)*H*-imidazo[1,2-*a*]pyridin-3-yl]-*N*,*N*-dipropylacetamide.

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doi:10.1016/j.ccr.2009.01.028

1. Historical survey

1.1. Discovery

Petsko [1], in an article about the Rosenberg's discovery of cisplatin says: "This is not a compound that would ever be found in any combinatorial library or collection of natural products. There isn't a single atom of carbon in it. No medicinal chemist would ever have thought of it. No targeted research program would have investigated it. No discovery-driven program of chemical genomics would have included it. Cisplatin ... came from a place no one would, at that time, have dreamt of looking-in for an anticancer drug."

In this review we will cover four aspects of this success story:

(i) a brief historical survey: from the early discovery of cisplatin to third generation platinum drugs;
(ii) an outlook to the burst of metal-drug research generated by this discovery;
(iii) the drug on route to the DNA: some intriguing questions;
(iv) the processing of Pt-DNA lesion: how subtle differences can result in dramatic effects.

Cisplatin was first synthesized by an Italian, Michele Peyrone, who graduated as a medical doctor in Turin in 1835 but soon afterwards (1839) abandoned medicine for chemistry and spent several years in different laboratories in France, Germany, Netherlands, Belgium, and Great Britain [2].

In the Liebig's laboratory, in Giessen, he synthesized (1845) a new platinum compound [3] containing two ammines and two chlorines, like the Reyset's salt [4], but having different physicochemical properties. There is no way to justify the obtainment of two different compounds assuming a tetrahedral geometry those days retained usual for tetravalent compounds. Some 50 years later, Alfred Werner proposed for these compounds a square planar geometry which can accommodate a *cis* and a *trans* isomer, the Peyrone's and Reyset's compounds, respectively (Fig. 1) [5]. It was by accident that Barnett Rosenberg, a microbiologist, while investigating the effect of an electric field on cell division in bacteria, produced some Peyrone's compound generated electrochemically from the platinum electrodes and the various components present in the cell culture medium [6–8]. The astonishing result that cisplatin completely inhibited the development of the solid Sarcoma—180 tumor in mice was published in Nature on April 26, 1969 [9,10]. Clinical trials started in 1971 [11] and approval from the Food and Drug Administration granted in 1978 [12].

Since then cisplatin has become one of the best selling anticancer drugs in the world. It is a complete cure for testicular cancer, one of the most effective against melanoma and non-small-cell lung carcinoma, and in combination therapy it is considerably active against ovarian cancer [13].

1.2. Second and third generation drugs

As other respected drugs, also cisplatin has generated a family with second and third generation representatives (Fig. 1). Carboplatin was approved in 1989 (ovarian cancer) [13]. A more stable leaving group than chloride (1,1-cyclobutane-dicarboxylate) lowers the toxicity without affecting the antitumor efficacy (it is devoid of nephrotoxicity and is less toxic to the gastrointestinal tract and less neurotoxic, myelosuppression and trombocytopenia are dose limiting). Oxaliplatin was approved in 2002, it is active in patients with colorectal cancer in combination with 5-fluorouracyl and leucovorin (www.cancerbackup.org.uk/). It is based on the 1,2-diamino cyclohexane carrier ligand and retains activity against some cancer cells with intrinsic or acquired resistance to cisplatin [13]. Oxaliplatin is one of the top ten selling drugs with world wide sales in 2005/6 financial year of ~1.6 billion US dollars [12].

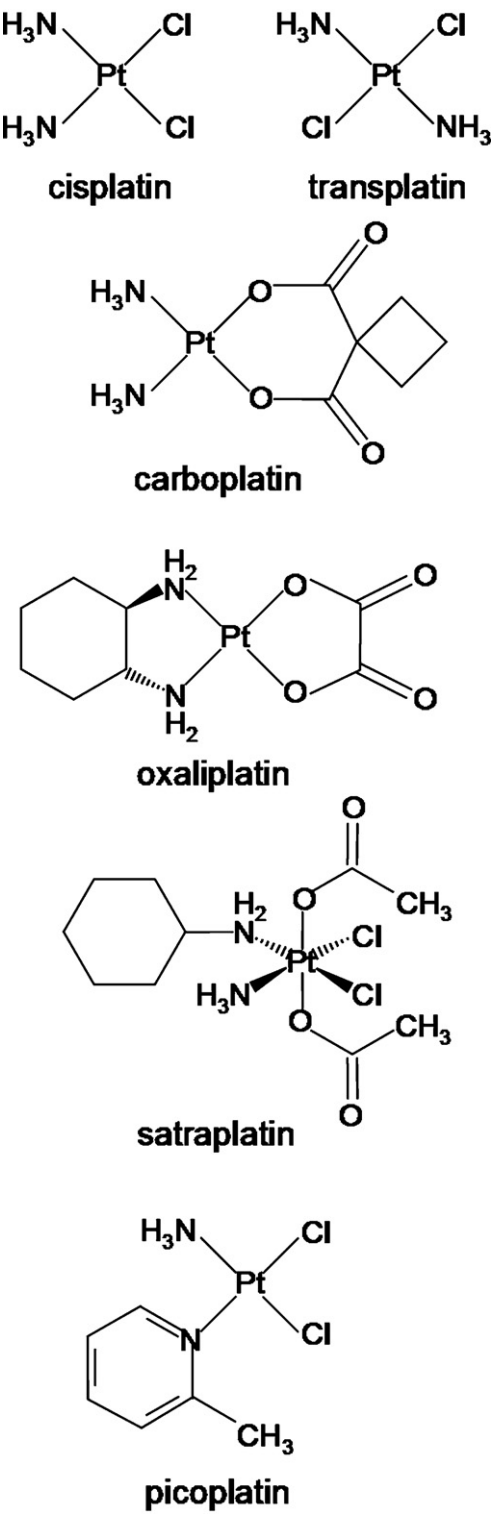


Fig. 1. Structures of Pt complexes, some are currently used in the clinics (cisplatin, carboplatin, and oxaliplatin) while others are in the pipeline (satraplatin and picoplatin). The inactive isomer of cisplatin, transplatin, is also reported.

Other platinum drugs are in the pipeline (Fig. 1): Picoplatin, differing from cisplatin for having a 2-picoline in place of an ammine ligand, is in Phase III clinical trials for small-cell lung cancer [14–17]. Satraplatin, differing from cisplatin for having a cyclohexylamine in place of an ammine ligand and two acetate ligands in axial positions

(it is a Pt^{IV} species), is under consideration by FDA for hormone-refractory prostate cancer. It is also orally active [18–21].

It should also be mentioned that platinum complexes with *trans* geometry or containing multiple platinum centers are undergoing extensive investigation and there is hope that, in the future, some of them can enter the clinical practice [22–25].

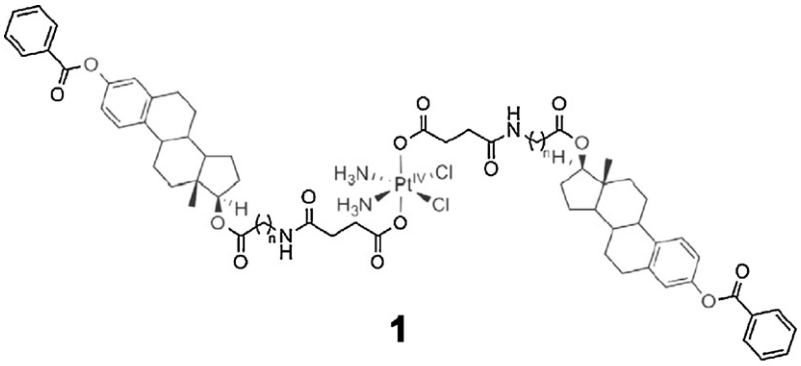
2. Manyfold possibilities of coordination compounds

2.1. Coupling with bioactive molecules and targeting to specific cancers

The countless chemical variations that a coordination compound (like cisplatin) can undergo have been highlighted in several excellent review articles [12,26–34].

Let us look, as an example, to the many possibilities provided by the addition to cisplatin of two extra ligands in axial positions, so generating a Pt^{IV} species. The two extra ligands stabilize the complex so minimizing the reaction with biomolecules in its way to the target. Moreover they give the possibility to tether to platinum other bioactive molecules (Fig. 2). Initially Lippard synthesized estrogen-tethered Pt^{IV} species targeted to estrogen receptor-positive breast cancer. Intracellular reduction releases one equivalent of cisplatin and two equivalents of estradiol. The latter induces upregulation of HMGB1, a protein which can increase cisplatin toxicity [35]. Dyson attached to platinum two molecules of ethacrinic acid, a diuretic in clinical use. Reduction to Pt^{II} in the cell results in the release of two equivalents of this potent inhibitor of glutathione-S-transferase (GST), an important enzyme which contributes heavily to drug resistance in several cancers [36]. Lippard also prepared a Pt^{IV} species, with succinic acid as axial ligand, capable of being tethered to an amine-functionalized single wall carbon nanotube. Each nanotube can load up to 65 platinum complexes, it penetrates cell membrane through clathrin-dependent endocytosis and, once in the endosome, the lower pH facilitates release of cisplatin by reduction and loss of the axial ligands [37]. Sadler has also pursued the strategy of a photochemical activation of Pt^{IV} drugs to realize active antitumor agents, rather than wait for a spontaneous intracellular chemical reduction [38–40].

Targeting to specific types of cancer cells or coupling with other bioactive molecules can also be pursued in Pt^{II} substrates. Let us mention the coupling of a cisplatin-like moiety with a Cu(3-clip-phen) residue reported very recently by Reedijk (Fig. 3). Cu(3-clip-phen) is a synthetic model of bleomycin, an antitumoral drug of natural origin usually complementary to cisplatin. The platinum metal center not only can act as a cytotoxic but also can anchor the Cu(3-clip-phen) moiety to DNA so favoring its strand-breaking activity [41]. We have tethered to a Pt^{II} moiety a ligand, TZ6, with high affinity for benzodiazepine receptors overexpressed in the central nervous system and in many tumor cancer cells (Fig. 4). After coordination to platinum the affinity for the receptor remains in the range of nanomolar and the selectivity for central over peripheral benzodiazepine receptors greater than 10,000 [42].



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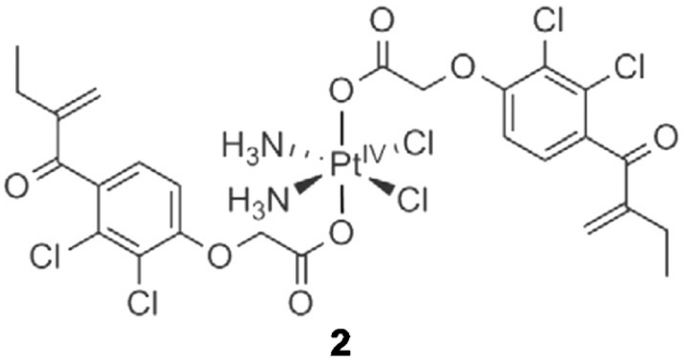

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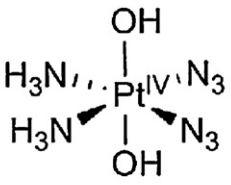

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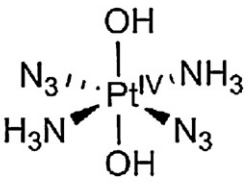

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Fig. 2. Platinum(IV) complexes that deliver cisplatin and two equivalents of estradiol (1) and the glutathione-S-transferase inhibitor ethacrynic acid (2). Photolabile platinum diazide complexes (3 and 4) are also shown.

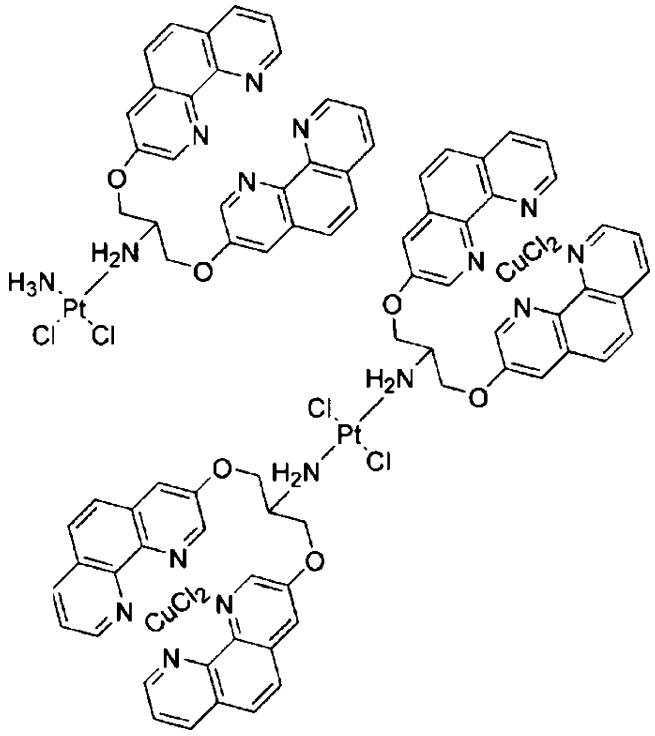

Fig. 3. Schematic representation of two active platinum-[(copper)-3-Clip-phen] complexes.

2.2. Relevance to other diseases

The same platinum, whose use up to now has been restricted to the treatment of some types of cancers, has a chance to provide valuable therapeutics also for other diseases. Meggers and colleagues have shown how platinum, similarly to other metal cores such as Os and Ru, can serve as a scaffold for building structures with defined three-dimensional shape which can mimic complex organic molecules, generally of natural origin, such as the indolocarbazole alkaloid staurosporine, a potent inhibitor of protein kinases (Fig. 5). The compound containing the two bissubstituted pyridines has the same affinity for the GSK-3α kinase as staurosporine (IC_{50} = 50 nM at 10 μM ATP concentration), in contrast the simple bispyridine complex turned out to be deprived of inhibitory activity [43]. Even if such platinum scaffold will not be suitable for clinical application, it certainly represents a potent tool for the exploration of the chemical space at the active site of kinases and enzymes in general.

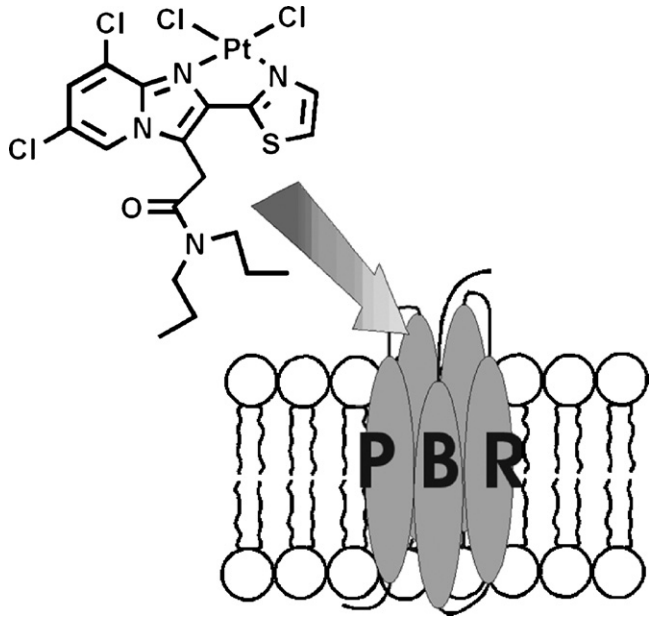

Fig. 4. Sketch of the platinum complex containing the TZ6 ligand having high affinity for benzodiazepine receptors. Reproduced from [42].

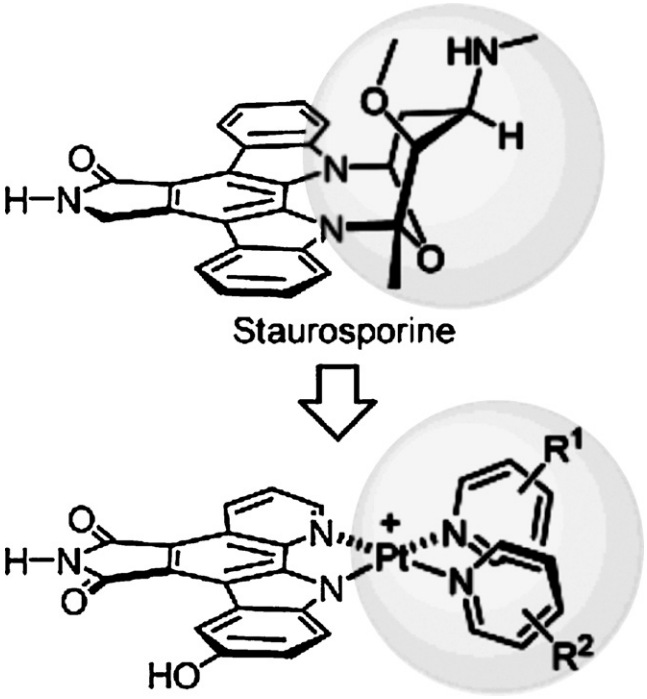

Fig. 5. Mimicking the overall shape of the indolocarbazole alkaloid staurosporine with a simple platinum complex. Reproduced from [43].

We wish to conclude this section by mentioning the recent paper by Barnham et al. showing how some platinum complexes we first synthesized over 10 years ago [44] and which contain, as well as cisplatin, two N and two Cl donor atoms are effective inhibitors of Amyloid β aggregation and could be used as therapeutic agents for Alzheimer's disease (Fig. 6) [45].

The current interest in platinum and other metal-based therapeutics is testified by the many articles appearing in Nature [13,46], Science [47], and many other journals and magazines and this notwithstanding the distrust of metals that still exists in many areas of pharmaceutical industry. From this point of view it is of particular value the fact that the National Institute of Health has recently launched a "Metals in Medicine" program.

3. The drug on route to DNA

3.1. Passive diffusion versus active transport

The development of cisplatin is often seen as the prototypical success story, however the precise mechanism of action still remains elusive [23].

A first simple picture describing the route of cisplatin from intravenous injection to nuclear DNA, acknowledged as the ultimate target, can be found in a text book by Lippard and Berg published some 15 years ago (Fig. 7) [48]. It contemplates: stability of cisplatin in the blood stream and in the extracellular fluids where the Cl^- concentration is high (>100 mM), entering the cell by passive diffusion, solvolysis in the cytoplasm favored by low Cl^- concentration (4–20 mM), finally positively charged solvato species reaching

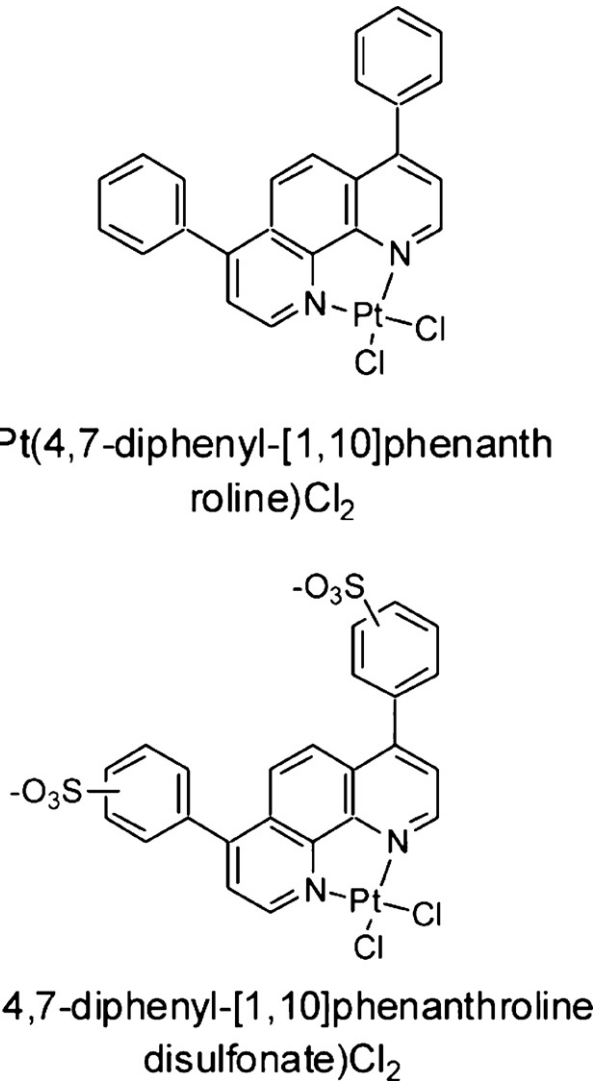

Fig. 6. Platinum complexes able to bind to amyloid-β (Aβ) inhibit neurotoxicity and rescue Aβ-induced synaptotoxicity.

the nucleus and reacting with DNA. It is estimated that only about 1% of intracellular cisplatin interacts with DNA [49,50] primarily by forming intrastrand cross-links between adjacent purines (over 90% of the total Pt-DNA adducts) [51–54]. This lesion is considered responsible for antitumoral activity [33,55–57].

However different opinions are also present in the debate and very recently there has been an editorial in AJP-Renal Physiology bearing the title: "Cisplatin induced cytotoxicity: is the nucleus relevant?". In this and related papers it is suggested that cisplatin toxicity could be initiated from the cytoplasm [50,58]. CDK2 activity (which normally promotes cell cycle progression) is important for promoting apoptosis in response to the cisplatin damage. The subcellular localization of CDK2 may determine which substrates are phosphorylated during cisplatin-induced apoptosis. It is also noted that irreversible binding of cisplatin to sulfhydryl groups of low- and high-molecular weight molecules correlates with a fall in the concentration of sulfhydryl moieties, especially in the mitochondrial and cytosolic fractions, resulting in the inhibition of a number of sulfhydryl-containing enzymes.

Let us address first the question how cisplatin can get in and out of the cell. For many years it has been taken for granted that cisplatin enters cells largely by passive diffusion [59–61]. With time, several evidences have accumulated suggesting that a number of active uptake and efflux mechanisms are also at play [62–66]. Moreover, altered regulation of these transporters can be responsible for the reduced accumulation in drug-resistant cells [64,67–69]. In Fig. 8 it is nicely shown how the platinum drug (displayed in green) is prevented from entering in resistant tumor cells [70,71].

A recent review by Gottesman summarizes the multiple pathways by which Pt-containing drugs can go into and out of the cell (Fig. 9) [72]. Apart from passive diffusion, a number of carrier-mediated import proteins have been identified [73], the main players being organic cation transporters [74–77], the copper influx transporter CTR1 [65,66,78], and an as-yet unidentified sodium-dependent process [79–84]. Endocytic routes, particularly macropinocytosis, may be involved as well [85,86].

Organic cation transporters appear to play a role even in the case of oxaliplatin whose activity towards the colorectal cancer appears to be due, at least in part, to the over-expression of such transporters in these cancer cells [74–76]. However the role of these transporters appears to be much greater in the case of cationic platinum complexes of formula *cis*-diammine(pyridine)chloridoplatinum(II) as nicely shown in a recently published paper by Lippard (Fig. 10) [77]. The activity of this type of platinum complexes towards murine tumor models was established long time ago by Hollis and coworkers [87,88]. We also contributed to this field reporting, formerly, an analogous compound with acyclovir, an antiviral drug, which was endowed with remarkable antitumor activity against Sarcoma—180 in mice [89–91], and, more recently, the complex with neocuproine and Me-cytosine which proved to be much more active than cisplatin against the whole set of 11 different human tumor cells with only one exception [92]. Moreover the greatest difference was observed for cells of the gastro-intestinal tract. Brabec and his group also investigated the interaction of the acyclovir-containing drug with DNA [90]. The structural and functional alterations disclosed by using biochemical methods were coincident with those reported in the very recent paper of Lippard which also includes a X-ray structure [77]. The prospects of this class of monofunctional Pt^{II} antitumor agents are nicely addressed in Lippard's paper.

3.2. The case of copper transporter 1

The involvement of CTR1 in the active transport of all three platinum drugs in clinical use has been extensively investigated by several groups. We wish to recall the excellent review of Safaei and Howell [65]. It has clearly been shown by biochemical techniques that: (i) CTR1 is a major determinant of the initial influx of platinum drugs when they are present at low, clinically relevant, concentrations; (ii) CTR1-mediated uptake of cisplatin requires endocytosis; and (iii) platinum-induced loss of CTR1 involves internalization from the plasma membrane by macropinocytosis followed by proteasomal degradation. Thus prevention of CTR1 degradation offers a potential approach to enhancing tumor sensitivity.

We also have started to investigate by NMR and other physicochemical techniques the interaction of CTR1 with platinum species [93]. CTR1 is a membrane protein, highly conserved from yeast to humans. It has a N-terminal extracellular domain carrying methionine-rich motifs, three transmembrane helices and an endocytic C-terminal region containing histidines and cysteines [94]. After coordination of copper the protein forms an oligomer which features a pore, containing methionines on its surface [94,95], through which copper(I) flows and reaches the intracellular C-terminus. Copper is then delivered to metal-transporting proteins called chaperones [96,97]. Different chaperones deliver copper directly to other proteins (such as SOD1) [98,99] or bring it to subcellular districts such as the mitochondrion [100,101] or the Golgi apparatus [102,103] to be loaded by other proteins there present (e.g. cytochrome *c* oxidase and ceruloplasmin, an iron oxidase, respectively) (Fig. 11) [104]. In all these steps the copper ion is

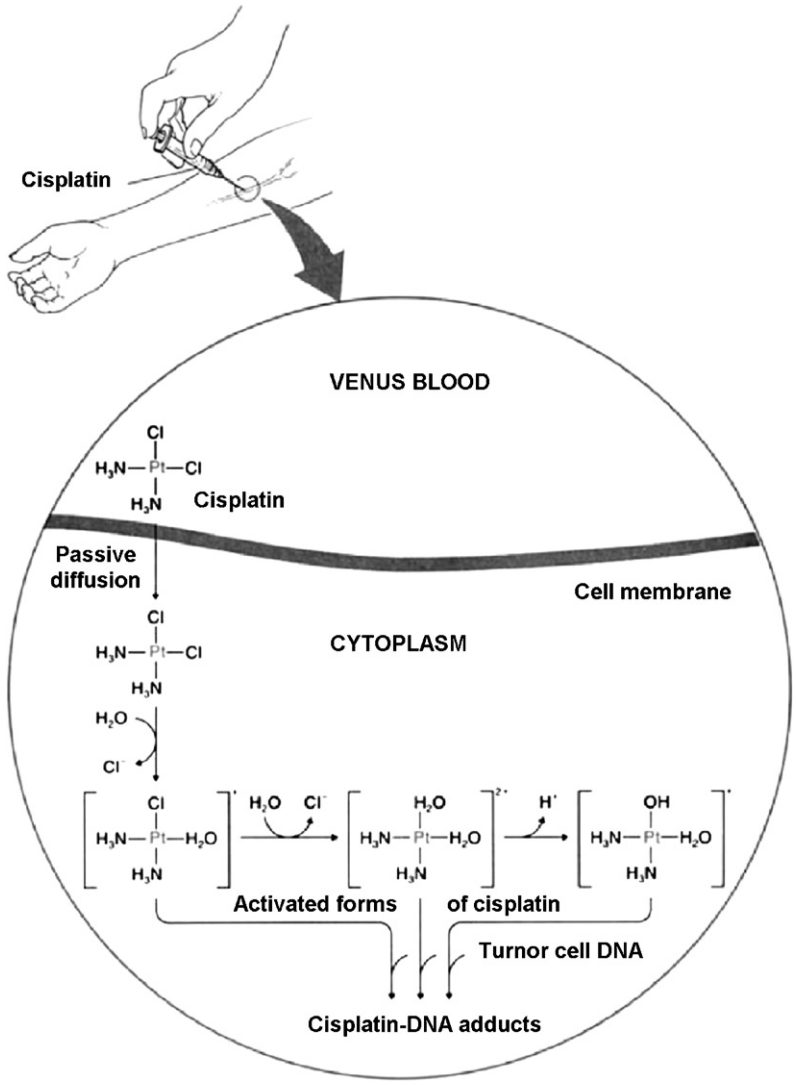

Fig. 7. Administration and *in vivo* chemistry of cisplatin. Reproduced from [48].

naked, but can platinum use the same pathway and keep the two ammine ligands? This was the question we wanted to address.

We chose one of the extracellular methionine-rich motifs of yeast CTR1 with slight modifications [105] to make it more amenable to water solution investigations (from now on Mets7) and performed the reaction with a selection of platinum complexes [93].

It was clear by ESI-MS experiments that cisplatin loses first one and then both ammine ligands and finally coordinates to Mets7 in the naked form. In contrast the antitumor inactive trans isomer

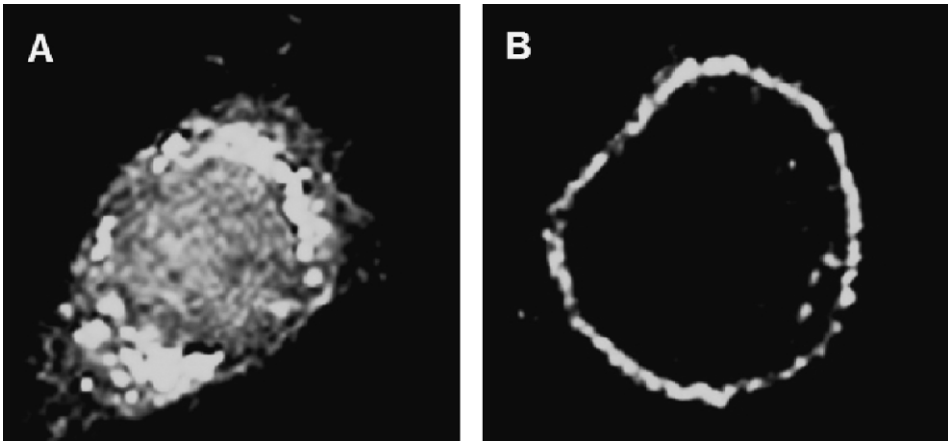

Fig. 8. Distribution of F-DDP (fluorescein-labeled cisplatin) in ovarian carcinoma 2008 cells (A) and the cisplatin-resistant subline 2208/C13*5.25 (B). Reproduced from [71].

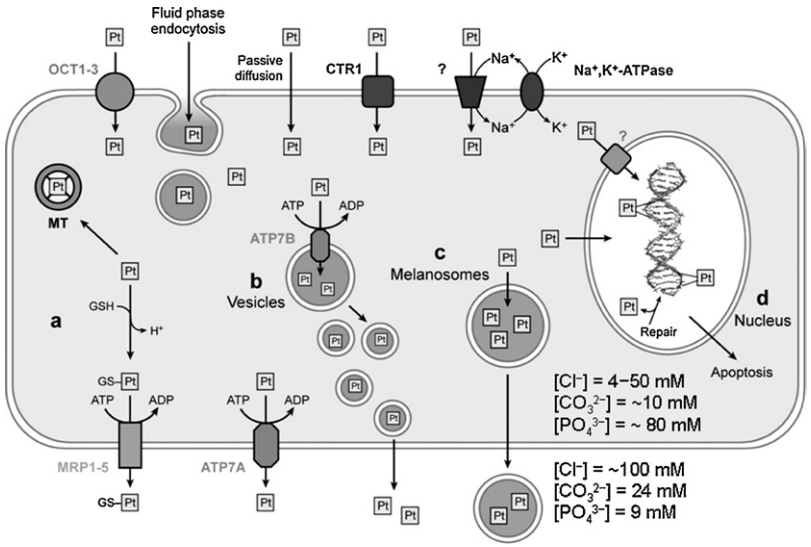

Fig. 9. Schematic representation of the mechanism affecting and controlling the cellular accumulation of platinum chemotherapeutics exemplified by cisplatin. Neutral platinum drugs can enter the cell by passive diffusion across the lipid bilayer or carrier-mediated import proteins like organic cation transporters (OCT) or copper transporter 1 (CTR1). Inside the cell platinum drugs can be deactivated by binding to thiol-rich metallothioneins (MT) or chelated by glutathione (GSH) and effluxed from the cell via the GS-X pumps (MRP1-5). Platinum drugs can also be ensnared in subcellular organelles such as vesicles via ATP7B influx, or melanosomes in melanoma cells, followed by exocytosis to expel platinum from the cells. Reproduced from [72].

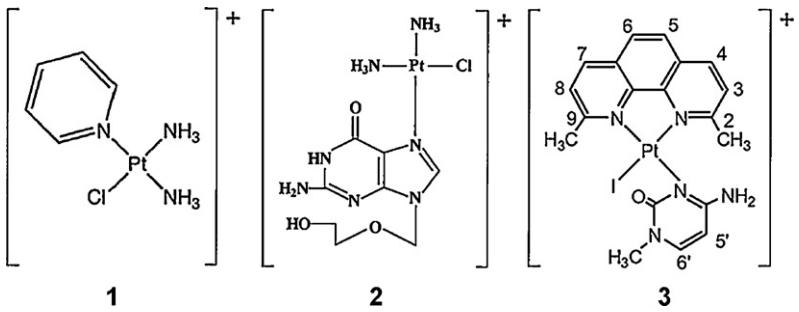

Fig. 10. Sketches of cationic complexes *cis*-diammine-pyridine-chlorido-platinum(II) (1, Hollis compound), *cis*-diammine-acyclovir-chlorido-platinum(II) (2), and *cis*-neocuproine-Me-cytosine-iodido-platinum(II) (3).

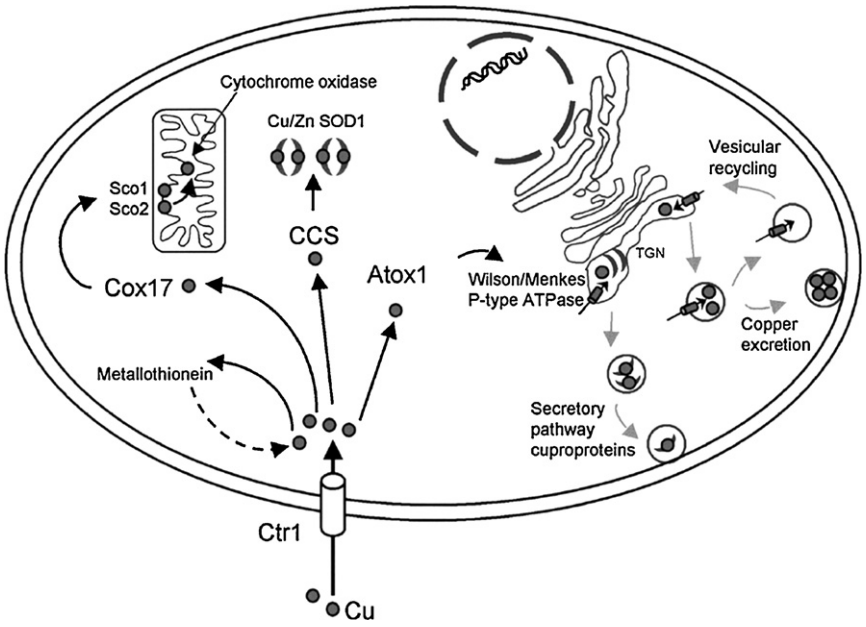

Fig. 11. Pathways of copper trafficking within a mammalian cell. The three principal chaperones, Cox17, CCS, and Atox1 are shown along with the respective protein targets, cytochrome *c* oxidase, SOD1 and the Wilson and Menkes transporting ATPases. Reproduced from [104].

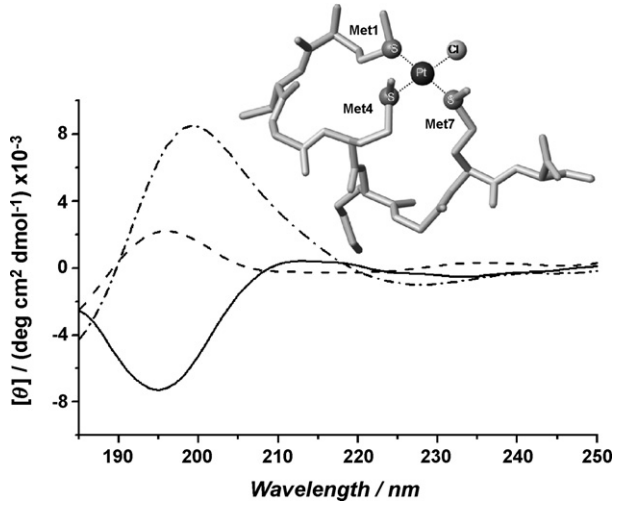

Fig. 12. Far UV spectra of Mets7 in the absence of platinum complex (solid line) and in the presence of cisplatin (dashed/dotted line) and transplatin (dashed line) 24 h after mixing. In the insert a structural model of the adduct between cisplatin and Mets7. Adapted from [93,106].

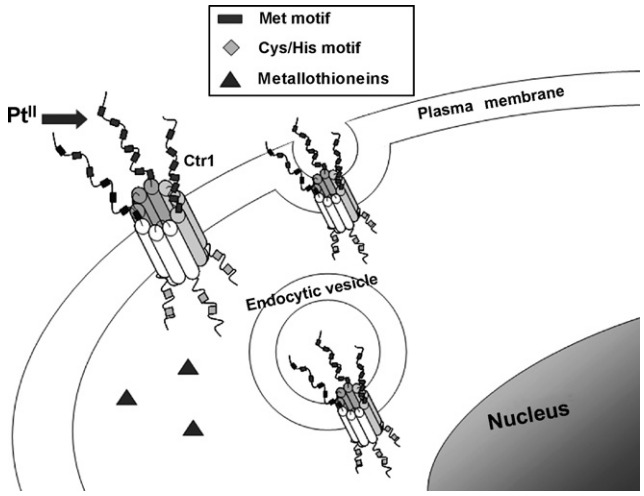

Fig. 13. Model of cisplatin trafficking mediated by yeast CTR1. The formation of endocytic vesicles may prevent drug inactivation by platinophiles. Reproduced from [106].

(transplatin) keeps the two ammines. The $^{1}H/^{13}C$ HSQC spectrum showed that both platinum complexes (cisplatin and transplatin) cause a remarkable downfield shift of the methyl and of the methylene group directly bound to the sulfur atom of all methionines, which is indicative of sulfur coordination to platinum. Moreover only in the case of cisplatin there was a significant downfield shift also for the β methylene protons of the methionines, which is indicative of a more dramatic change in conformation. The loss of the two ammine ligands only in the case of cisplatin was also confirmed by $^{1}H/^{195}Pt$ HMQC spectra showing only cross-peaks between platinum and methionine protons in the case of cisplatin and cross-peaks between platinum and methionine and ammine protons in the case of transplatin. Coordination of sulfur and loss of the ammines in the case of cisplatin also causes a dramatic upfield shift of the platinum resonance [93]. The change in peptide conformation, consequent to platinum coordination, was investigated by circular dichroism. The Cotton feature switches from that typical of a random coil for free Mets7 to that typical of a β-turn after reaction with cisplatin. A modeled structure of the adduct is given in Fig. 12 [106]. Reaction with transplatin leads to a Cotton feature which is half way in between that of free Mets7 and that of the adduct with cisplatin.

There appears to be a perfect parallelism between copper and platinum; in both cases the metal ion is taken up by methionine-rich motifs in the naked form. But if this is fine for copper, which then is taken up by chaperones, it is not useful for platinum which must keep the ammine ligands to remain active [33,55–57]. For antitumor activity a naked platinum ion would be useless. What can we imagine?

As for copper, also for platinum there could be an alternative mechanism for the uptake, that is a pinocytosis with formation of an endocytic vesicle which incorporates a portion of external solution with all chemicals there present including active cisplatin [66,107]. A vesicle trafficking would deliver cisplatin to subcellular districts, including the nucleus, while protecting the drug from attack by cytosolic platinophiles such as metallothioneins and glutathione to mention the most common (Fig. 13) [93,108]. However this attractive hypothesis requires further experimental support.

Warnings about the possible interference of platinophiles have accompanied the whole research history of antitumoral cisplatin [109–112]. The reaction of metallothioneins with several platinum complexes has also revealed that, while cisplatin readily loses all ammine ligands, inactive transplatin keeps both ammines notwithstanding the fact that these proteins are highly rich in sulfhydryl groups (Fig. 14) [113]. Cisplatin loses immediately one ammine ligand even reacting with cytochrome *c* which contains a single sulfur ligand (a methionine) on its surface [114]. Several authors have stressed the fact that, given the strong thermodynamic preference of Pt for S-donor ligands and the presence of so many cellular platinophiles in the cytosol, one would predict cisplatin never reach DNA [23]. Thus the hypothesis of cisplatin delivery by vesicle trafficking could also account for its survival in the very risky cytosol.

4. Cellular response to platinum-induced DNA damage

4.1. Proteins that specifically recognize distorted DNA

Somehow ca. 1% of total intracellular platinum reaches the nucleus [49,50] and gives mainly the intrastrand cross-link with adjacent purines of DNA [51–54]. The cellular response to platinum-induced DNA damage is a rather complex question addressed by many laboratories and reviewed in several articles [57,115–118]. The overall picture, as summarized in a recent article by Lippard, is reported in Fig. 15 [33]. A variety of cellular proteins specifically recognize duplex DNA distorted by platinum cross-links. These proteins include those involved in repair processes, proteins containing HMG domains, and many others [116–121]. HMGB1 is one of the early proteins discovered to bind cisplatin-modified DNA [122,123], it is an abundant non-histone chromosomal protein ($\sim 10^6$ copies per cell) which regulates numerous nuclear functions including transcription, replication, recombination, and general chromatine remodeling [124,125]. This 30 kDa protein of 215 aminoacids comprises two HMG box domains, A and B, and an acidic C-terminal tail. One hypothesis is that HMGB1 shields damaged DNA inhibiting Nucleotide Excision Repair (NER) [57]. Another possibility, called 'titration' model, is that, because of binding to damaged DNA, the protein molecules are removed from their physiological places impairing vital cell functions [57]. Domain A interacts more strongly with 1,2-intrastrand cross-linked DNA than domain B [126,127]. The stability constant for domain A depends from the flanking nucleotides in the DNA and the spectator ligands in the platinum complex [126,128]. A crystal structure of domain A bound to a 16-bp DNA duplex containing in the middle a cisplatin 1,2-d(GpG) cross-link was determined by Lippard et al. [129]. The HMGB1 full

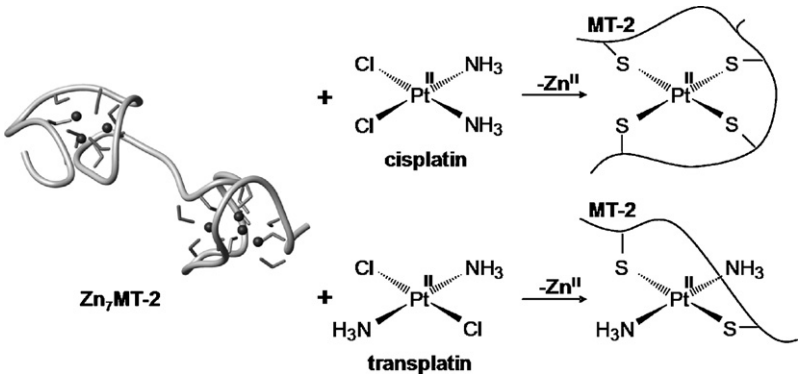

Fig. 14. Reaction of human metallothionein Zn_7MT-2 with cisplatin and transplatin. Reproduced from [106].

length protein recognizes cisplatin 1,2-intrastrand [123,130] as well as interstrand cross-links and the interaction is unaffected by the sequence context [131].

4.2. Different processing of DNA adducts with enantiomeric platinum drugs

We wanted to investigate if, on the basis of present knowledge of cellular response to platinum-induced DNA damage, it was possible to explain an early observation concerning a striking difference in the mutagenic activity of cisplatin-analogues containing *R,R* or *S,S*-1,2-diaminocyclohexane (DACH, the same carrier ligand of oxaliplatin) in place of the two ammines. The Ames' test [132] was used by us to show that the *S,S*-enantiomer was far more mutagenic than the *R,R*-enantiomer (the one approved for medical use) or the meso form towards several strains of *Salmonella typhimurium*. The difference in mutagenicity between the two enantiomers was ten or even hundred times [133]. Moreover this striking behavior did not apply only to the diaminocyclohexane complex but also to other complexes with analogous chiral diamines such as 2,3-diaminobutane (DAB).

Certainly there must be something different in the interaction of platinum complexes with enantiomeric ligands and DNA. This issue has been addressed by different groups and using different techniques but the results have been rather inconclusive [134,135]. We believe the reason is that the site of the intrastrand cross-link is rather dynamic and, as such, it eludes a precise characterization performed either by X-ray or by NMR. In the case of X-ray intermolecular interactions can play a role while in the case of NMR the resulting structure can be the average of several conformations in rapid interconversion. For instance, the great dispersion observed in the values of the dihedral angle between the cross-linked guanines, which span all values between 25° and 75°, is not realistic and could be just a consequence of the inadequacy of the experimental tools [136]. Thanks to the collaboration with the group of prof. Brabec, in Brno, chemical probes were used for sensing the different distortion induced in DNA by complexes with enantiomeric configurations of these amines (either DAB or DACH).

The DAB compounds were the first to be investigated. While bending and unwinding of DNA cross-linked at two adjacent guanines of the same strand are very little affected by the nature of the bases flanking the cross-link in the case of cisplatin [137], this is not the case for complexes with bulkier carrier ligands such as DAB. In particular, by exploring different sequences, it was found that TGGT is very sensitive to the chirality of the diamine, bending and unwinding being far less pronounced for the *S,S*-enantiomer [138]. Even more significant was the overall distortion at the site of the lesion as revealed by chemical probes. These are chemical reagents specific for individual nucleobases which are not perfectly paired with the complementary base but somewhat exposed to the solvent. After reaction with the chemical reagent, treatment with a base (piperidine) causes strand break and the different fragments can be analyzed by gel-electrophoresis. From the size and abundance of the fragments can be deduced the site and extent of distortion. The overall distortion was similar for the two enantiomers in the CGGA sequence but very different in the case of the TGGT sequence [139]. A clash between a Me group of the DAB ligand and the methyl of thymine in 5′-position appears to be responsible for such a difference in distortion [138].

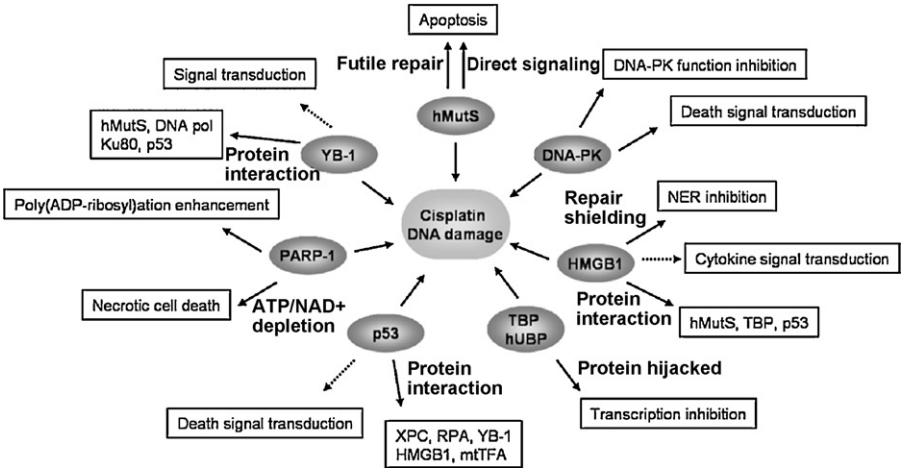

Fig. 15. Roles of proteins that bind to DNA following cisplatin damage. Reproduced from [33].

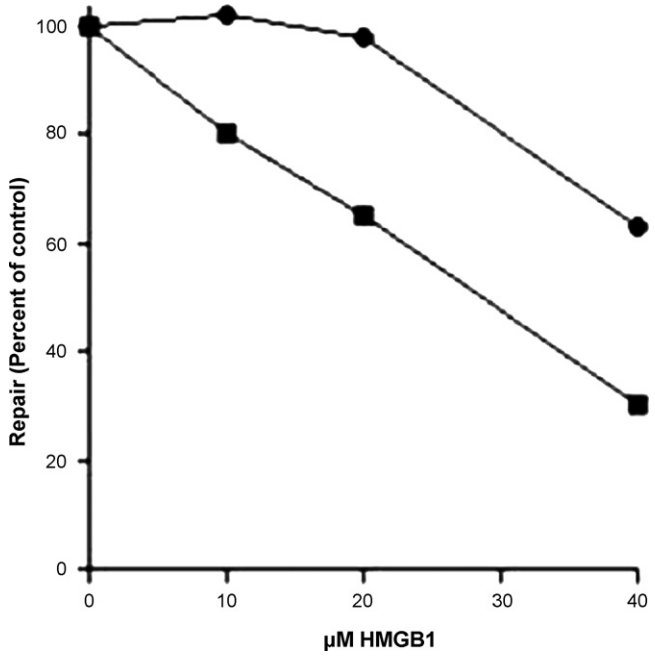

Fig. 16. Plot of nucleotide excision repair (NER) performed on 148 base-pair substrates containing a single central cross-link of Pt(*R,R*-DAB) or Pt(*S,S*-DAB) and incubated with the indicated concentration of HMGB1 for 1 h on ice under NER assay conditions prior to the addition of the rodent excinuclease and further incubation for 40 min. Reproduced from [140].

The further step was to see if the different distortion could influence the interaction with proteins, in particular the HMGB1 protein. The results were remarkable. The protein gives a much stronger interaction with DNA modified by *R,R*-DAB than with DNA modified by the *S,S*-enantiomer. Moreover the interaction with *R,R*-DAB-modified DNA is even stronger than that with cisplatin-modified DNA [140].

Finally it was checked to see if this protein could interfere with NER and the answer was yes. In the case of *R,R*-DAB-modified DNA there is a linear inhibition of repair as a function of protein concentration, in contrast in the case of the *S,S*-enantiomer there is no inhibition up to a protein concentration of 20 μM (Fig. 16). Therefore one can hypothesize that the *R,R*-enantiomer, the one present in oxaliplatin, forms DNA lesions which are more difficult to repair and therefore it is more cytotoxic. In contrast, the *S,S*-enantiomer gives lesions that can be more easily repaired but with an error-prone mechanism and therefore results to be much more mutagenic [140].

More recently the investigation has been extended to the DACH complexes. As anticipated, the behavior was superimposable to that already described for DAB complexes: (i) marked difference between the two enantiomers for the TGGT sequence, the behavior of the complex with *R,R*-configuration of the diamine (that of oxaliplatin) being more similar to that of cisplatin [141]; (ii) the HMGB1 protein interacting more strongly with *R,R*-DACH-modified DNA than with *S,S*-DACH-modified DNA. The effect of interaction with HMGB1 upon NER has not yet been verified but we are confident that what has been observed for the DAB complexes also applies to the DACH species.

In the case of DACH compounds the translesion replication capacity (TLS) of the exonuclease-deficient Klenow fragment of DNA polymerase I was also investigated. Importantly, in all sequence contexts the TLS was greater for the template containing the cross-link of cisplatin than for those containing the cross-link of DACH. There is again something peculiar about the TGGT sequence for which the TLS is significantly greater than for the other sequence contexts and the *R,R*-enantiomer conforms more to the behavior of cisplatin than the *S,S*-species [141]. It is likely that the bulkier DACH ligand can interfere more strongly with the conformational requirements of the polymerase at the closed catalytically active ternary complex DNA-polymerase-dNTP than cisplatin's ammine groups.

The detailed mechanism of transcriptional stalling at cisplatin-damaged DNA [142] and of error-prone bypass of DNA lesions by DNA polymerase η [143] have been described in two recent articles in Nature and Science. These results also give some hints for understanding the different behavior of *R,R* and *S,S*-DACH compounds.

Always looking for differences between the two enantiomers the interstrand cross-links were also investigated. The cross-links of opposite strands are usually much fewer than those within the same strand, but their relative efficacy remains unknown. Bending and unwinding are definitely greater for DACH compounds than for cisplatin. Moreover the distortion induced by the *R,R*-enantiomer is more similar to that induced by cisplatin while that induced by the *S,S*-enantiomer expands over a larger number of base-pairs [144]. Therefore also for the cross-links of opposite strands there are differences between *R,R* and *S,S*-DACH compounds, however, as already anticipated, their role in determining the fate of the cell remains still unraveled.

5. Concluding remarks

In conclusion, that of cisplatin has been, beyond doubt, a success story. The huge number of patients that have been completely cured after cisplatin treatment of cancer speaks as such. The precise mechanism remains elusive, but we have to consider that the scientific progress has always been marked by uncertainties. Nevertheless cisplatin and its several analogues have provided a fertile ground for exciting (bio)chemistry and the enormous progress made in understanding cisplatin reactivity towards biomolecules and the tumor cell physiology will not be without effect upon treatment of cancer and other diseases.

We have also learned that coordination chemistry in biological systems is more than just a matter of bond formation and stability as molecular recognition also has a profound effect. This has forced inorganic chemists to address systems covering different ranges of complexity, from simple coordination compounds to biomolecules made of thousands of atoms, from individual molecules to large assemblies. Each time there have been difficult experimental and theoretical problems to be solved, each time a new exciting landscape has arisen.

Acknowledgments

The authors thank the University of Bari, the Italian Ministero dell'Università e della Ricerca (PRIN 2005032730), the Consorzio Interuniversitario di Ricerca in Chimica dei Metalli nei Sistemi Biologici (CIRCMSB), and EC (Cost Action D39) for support. We gratefully acknowledge Dr. Francesco Cannito of CIRCMSB for assistance in manuscript preparation.

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