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Chiara Gabbiani

Proteins as possible targets for antitumor metal complexes

Biophysical studies of their interactions

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Chapter 1

Introduction

1. Metal complexes with pharmacological properties

The importance of metal compounds in medicine dates back at least to the 16th century with reports on the therapeutic use of metals or metal containing compounds in the treatment of cancer. The pharmaceutical use of metal complexes has indeed an excellent potential. A broad array of medicinal applications of metal complexes have been investigated, and several recent reviews summarize advances in these fields. Now the list of therapeutically prescribed metal containing compounds includes platinum (anticancer), silver (antimicrobial), gold (antiarthritic), bismuth (antiulcer), antimony (antiprotozoal), vanadium (antidiabetic) and iron (antimalarial). Designing ligands that will interact with free or protein-bound metal ions is also a recent focus of medicinal inorganic research. For example, chelating ligands for copper and zinc are being investigated as a potential treatment for Alzheimer's disease. Metal ions are electron deficient, whereas most biological molecules (proteins and DNA) are electron rich; consequently, there is a general tendency for metal ions to bind to and interact with many important biological molecules. In addition, metal ions usually manifest a high affinity for many small molecules, e.g. O_2, that are crucial for life. These considerations alone have fuelled much of the past and current interest in developing novel means to use metals or metal containing agents to modulate biological systems.

Developing metal complexes as drugs, however, is not an easy task. Accumulation of metal ions in the body can lead to very deleterious effects. Thus, biodistribution and clearance of the metal complex as well as its pharmacological specificity have to be carefully considered. Favourable physiological responses of the candidate drugs need to be demonstrated by in vitro studies with targeted biomolecules and tissues as well as in vivo investigation with xenografts and animal models before they may enter clinical trials. A mechanistic understanding of how metal complexes produce their biological activities is crucial to their clinical success, as well as to the rational design of new compounds with improved potency.

1.1 Metal-based anticancer drugs

Medicinal inorganic chemistry offers new possibilities for the treatment of cancer, a filed that has traditionally been dominated by antitumor drugs based on organic and/or natural products chemistry. The development of metal-based antitumor drugs has been

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stimulated by the success of cisplatin, cis-[diamminedichloroplatinum(II) and carboplatin], today the world's best selling anticancer drugs .

Following the serendipitous discovery of the antitumor properties of cisplatin by Rosenberg and co-workers,1 some platinum complexes with reduced toxicity (carboplatin) and activity against cisplatin resistant (oxaliplatin) or sensitive (nedaplatin) tumors have been discovered and now they are worldwide used in clinical protocols. In addition, some Pt(IV) antitumor drugs have good prospects for oral administration so that the quality of life of cancer patients could be improved avoiding the upsetting effects of intravenous administration of platinum drugs.

Although platinum complexes are the metal complexes most intensively investigated as antitumor drugs, research has been extended to other metals. In fact, platinum group metals and non-platinum group metals have shown antitumor activity in experimental tumors in animals and even in man. Among non-platinum antitumor metal complexes there are two groups of compounds which deserve special attention. These are ruthenium and gold complexes.

Contrary to cisplatin, the Ru(III) complex called NAMI-A has a unique feature, namely a higher activity against metastases than against primary tumors. At variance with platinum complexes, it is noteworthy to mention that DNA seems not to be the primary cellular target mediating the antitumor activity for most non-platinum metal-based drugs.

1.1.1 Cisplatin and related anticancer drugs

The interest in platinum-based antitumor drugs has its origin in the 1960s, with the serendipitous discovery by Rosenberg of the inhibition of cell division induced by Pt complexes.1

Thousand of platinum compounds have been prepared thereafter and evaluated as potential chemotherapeutic agents, although few have entered clinical use.

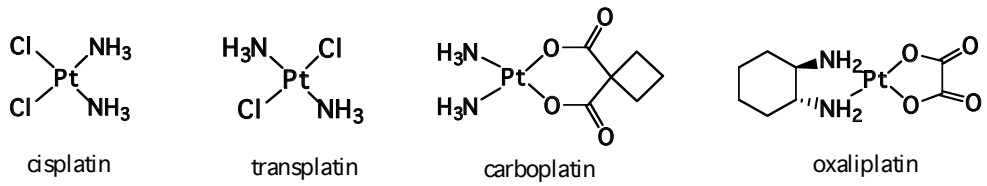


Figure 1.1 Chemical formulae of classical platinum compounds.

Cisplatin, is effective in treating a variety of cancers, especially testicular cancer, for which it has a greater than 90 % cure rate. It is generally believed to exert its anticancer effects by interacting with DNA and inducing programmed cell death (apoptosis). Following intravenous administration, cisplatin encounters a relatively high chloride concentration in the blood (approximately 100mM) that limits replacement of its

chloride ligands by water molecules (that is, the aquation process is prevented).13 However, cisplatin is vulnerable to attack by proteins found in the blood plasma, particularly by those that contain thiol groups, such as human serum albumin (hSA). In fact, studies have shown that one day after cisplatin administration, 65-98 % of the protein in the blood plasma is protein bound. This protein binding has been blamed for deactivation of the drug and for some of the severe side effects of cisplatin treatment.

The intact cisplatin can enter tumor cells, mainly by passive diffusing through the cell membrane, although facilitated or active transport mechanism may contribute to the cellular uptake as well. The intracellular chloride concentration is relatively low (approximatively 4-20 mM); hence one of the chloro ligand of the intact complex is rapidly replaced by water, forming a reactive, positively charged species that cannot readily leave the cell. This monoaquated platinum species reacts with one of the DNA bases, usually guanine, forming monofunctional DNA adducts.

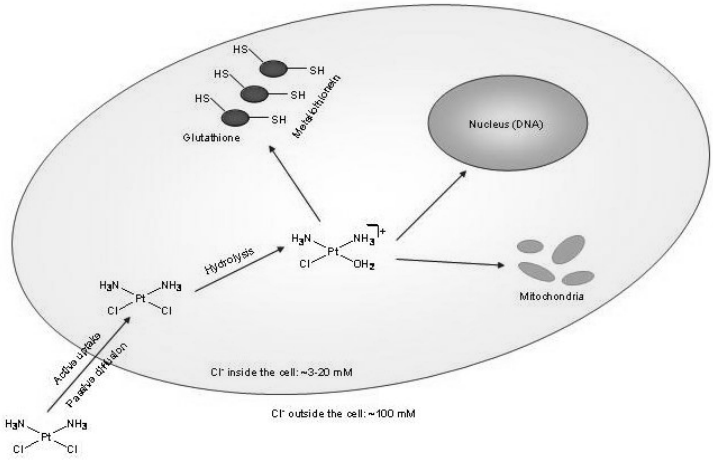


Figure 1.2 The cellular uptake of cisplatin and its targets.

Ring closure to form a bifunctional adduct with the nucleic acid may occur either directly from the monofunctional adduct or may involve aquation of the second chloro ligand followed by rapid ring closure.

The cytotoxicity of cisplatin originates, therefore, from its binding to the DNA double helix and the consequent formation of covalent cross-links, which cause significant distortion of helical structure and results in inhibition of DNA replication and transcription.

However, the effectiveness of cisplatin treatment is limited by the phenomenon of tumor resistance. Several tumors are intrinsically resistant to cisplatin (e.g. colon cancer, non-small-cell lung cancer), while others acquire resistance after exposure to the drug over time. The cellular mechanisms of cisplatin resistance have been identified as

decreased intracellular accumulation, strong binding to inactivating sulfur-containing molecules inside the cell, and increased repair of platinated DNA by enzymes .

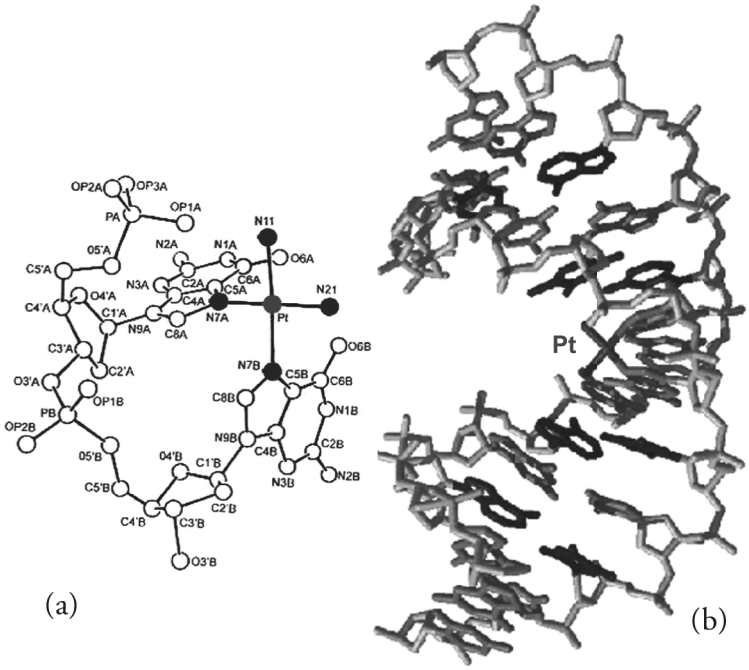


Figure 1.3 (a) Structure of cisplatin coordinated to two guanines in a DNA duplex. (b) Structure of cisplatin coordinated to a dinucleotide containing two guanines. Notice the destacking of guanine bases, which would normally be parallel to one another.

If cisplatin cannot accumulate in the cell, it cannot reach the DNA found inside the cell, bind the DNA, and cause cell death. Therefore, it is beneficial for the cancer cell to develop mechanisms either to keep cisplatin out of the cell or to remove cisplatin from the cell; indeed, reducing cisplatin accumulation by cancer cells seems to be a major form of acquired resistance. The mechanism of decreased intracellular accumulation of cisplatin is not well understood, but it seems that the cell has some control over cisplatin entering the cell. This suggests that cisplatin does not enter the cell by passive diffusion alone but that there is some active transport system involved. Furthermore, additional experiments have shown that decreased cellular accumulation of cisplatin is not due to increased efflux of cisplatin.

Once inside the cell, cisplatin can interact with a variety of other molecules besides DNA including two major sulfur-containing biomolecules, metallothionein and glutathione, that sequester cisplatin and remove it from the cell. Metallothionein (MT) are low molecular mass cysteine-rich proteins and are believed to be involved in intracellular detoxification of metal ions. Production of MT is triggered by the presence of heavy metal ions, glucocorticoids (which are steroid hormones that promote the formation of both glucose from non-carbohydrate sources and glycogen, enhance the degradation of fat and protein, and enable animals to respond to stress), interferon (which

is a signaling molecule in the immune system that greatly enhances antiviral responses) and stress. Cisplatin administration leads to the induction of MT in, e.g., the liver that may bind and inactivate platinum ions. Reactions between MT and cisplatin lead to displacement of the amino ligands and give rise to [PtS4] clusters containing 7-10 platinum atoms per mole. MT may contribute to cisplatin resistance, but the results are inconclusive. In some cases, the levels of MT are higher in cisplatin-resistant cells, but in other cases, the MT levels are unaffected. Trans-DDP reacts with MT faster than cisplatin and this may be one of the possible causes of its inactivity.

Like metallothionein, glutathione is also involved in detoxification. GSH reacts with hydrogen peroxide and organic peroxides, the harmful bioproducts of aerobic life; GSH is also essential for maintaining the normal structure of red blood cells. The binding of platinum(II) to S-thiolates tends to be irreversible, in contrast to S-thioethers. Reaction between cisplatin and GSH forms a 2:1 (GSH:Pt) complex which may therefore inactivate the drug and be a part of cellular resistance mechanism. Moreover, there is an over-expression of the pump for glutathione conjugates in cisplatin resistant cells, suggesting that Pt-GSH complexes are pumped out of the cell. Again, like MT, levels of GSH are increased in some, but not all, cisplatin-resistant cells, suggesting that there are other mechanisms of cellular resistance.

For cisplatin, binding to methionine is normally considered as an inactivating step. The metabolite $[Pt(Met)_2]$ has been detected in the urine of patients treated with cisplatin and it is a relatively unreactive complex, existing in solution as a mixture of three diastereoisomers of each of the two geometrical isomers. However, in the last years, it has been suggested that Met could play a secondary, but at least important, role in DNA platination by platinum-based drugs even if this hypothesis is yet not completely proved.

Another way that cells can become resistant to cisplatin is to have an enhanced ability to remove cisplatin-DNA adducts and to repair cisplatin-induced lesions in DNA.

Such an ability might result from the presence of specific DNA repair proteins.

Therefore, the clinical success of cisplatin is limited by significant side effects and acquired or intrinsic resistance. Thus, research has focused much attention on designing new platinum compounds with improved pharmacological properties and a broader range of antitumor activity. In general, for a platinum drug to gain clinical approval, it must posses at least one distinct clinical advantage over cisplatin. Such advantage may include: activity against cancers with intrinsic or acquired resistance to cisplatin treatment, reduced toxic side effects, or the ability to be administered orally. Several platinum complexes are currently in clinical trials, but these new complexes have not yet demonstrated significant advantages over cisplatin.

Following the initial studies on cisplatin, a number of studies were performed to determine the structural features required to endow a platinum(II) compound with antitumor activity. Strategies for developing new platinum anticancer agents, usually cisplatin analogues, include the incorporation of carrier groups that can target tumor cells with high specificity. Targeting platinum anticancer compounds to specific cell types may increase cytotoxic activity, reduce unwanted side effects, and diminish resistance due to limited uptake. These complexes may provide a broader spectrum of antitumor activity.

Two main second generation platinum anticancer drugs are carboplatin and oxaliplatin. Carboplatin, [cis-diammine-1,1-cyclobutanedicarboxylate-platinum(II)] (CPT), is a platinum analogue structurally related to cisplatin. Notably, replacement of two chloride groups by the bulkier cyclobutane dicarboxylate bidentate ligand, while not changing the overall charge of the complex, renders the aquation process of carboplatin far slower than in the case of cisplatin. The rate constant for the hydrolysis of carboplatin is very low, with a ks value of 8.14×10^{-8} s^{-1}; this means that, after one month at 25 °C, the decrease of carboplatin concentration is expected to be lower than 2% of its initial value. , At variance, the rate constant for the hydrolysis of cisplatin is ks= 6.32×10^{-5} s^{-1}, with a half life of 2.5 h at 37 °C, thus at least 300 times faster.

In spite of its extreme and widely documented in vitro kinetic inertness, CPT still behaves in vivo as an effective cytotoxic and antitumor agent, manifesting a more favourable toxicological profile than cisplatin itself. The spectrum of the biological actions of CPT substantially reproduces that of cisplatin implying an identical mechanism of action. Thus, its outstanding pharmacological actions must necessarily depend on specific activation processes taking place within biological fluids.

These interactions are believed to display some selectivity thus attenuating the systemic toxic effects of carboplatin. Many previous studies have revealed that carboplatin activation might arise from a "ring-opening" reaction of the cyclobutanedicarboxilate ligand. Indeed a ring opening process is readily detected during the reactions of carboplatin with small biomolecules or peptides containing sulphur, such as methionine residues.

Oxaliplatin, (trans-L-diaminecyclohexane) oxalatoplatinum(II)), was approved in France, in the United Kingdom, and other European countries in 1996 and in the United States in 2002 for clinical use against advanced colorectal cancer. It is the only platinum compound to have displayed activity against colorectal cancer so far. The chemical structure of oxaliplatin differs from those of cisplatin or carboplatin by the attachment of a cyclohexane ring to the nitrogen atoms. It is a compound with relatively good water solubility and achieved good efficacy in experimental tumour models and usually displayed low haematological toxicity and minimal or no nephrotoxicity in preclinical studies.

Oxaliplatin shows similar chemical behaviour and has a comparable mechanism of action as compared to the other platinum derivatives. First, the pro-drug oxaliplatin is activated by conversion to monochloro, dichloro and diaquo compounds by non-enzymatic hydrolysis and displacement of the oxalate group. The kinetics of hydrolysis differ among platinum compounds, being slower for oxaliplatin than for cisplatin. The highly reactive monochloro, dichloro and diaquo intermediates react with sulphur- and amino groups in proteins, RNA and DNA. Its anti-tumour effects are thought to be related to the formation of Pt–DNA adducts. Other reactions include irreversible binding to biomolecules such as albumin, cysteine, methionine and reduced glutathione, which are in fact the first steps of in vivo biotransformation and cellular detoxification.

Recent attention has also focused on trans-platinum complexes, polyplatinum compounds, and platinum(IV) analogues.

The original empirical structure-activity relationships considered the trans Pt complexes to be inactive. However, several groups have shown that some trans compounds are active in vitro and in vivo. An important difference between cisplatin and its trans analogue, transplatin, is that the latter is kinetically more reactive than cisplatin and more susceptible to deactivation. Careful drug design using a sterically hindered ligand may reduce the kinetic reactivity of trans Pt complexes.

As the trans isomer forms different Pt-DNA adducts than cisplatin analogues, it is hoped that trans Pt complexes could overcome cisplatin resistance in certain tumors. Several groups have pursued this concept of activating the trans geometry. For example, the antitumor activity of trans Pt complexes have also been demonstrated by Natile and co-workers that have developed a series of compounds with iminoether ligands. Both the cis and trans isomers of this family, react slower with DNA than cisplatin and transplatin.

This was attributed to the greater steric hindrance introduced by the iminoether ligands.

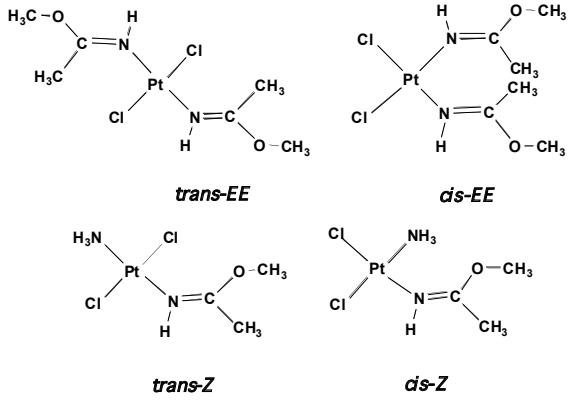


Figure 1.4 Schematic drawing of the selected platinum(II) iminoether complexes.

Also several differences in cytotoxic activity have been observed between trans and cis isomers. For example, in P388 leukemia-bearing mice, the trans-EE (trans-$[PtCl_2\{(E)\text{-}HN{=}C(OCH_3)CH3\}_2]$) isomer showed greater antitumor activity than the cis-EE (cis-$[PtCl_2\{(E)\text{-}HN{=}C(OCH3)CH_3\}_2]$, respectively) compound.

Also, trans-EE exhibited in vivo activity against an acquired cisplatin-resistant P388 cell line, whereas cis-EE was inactive.

1.1.2 Ruthenium anticancer agents

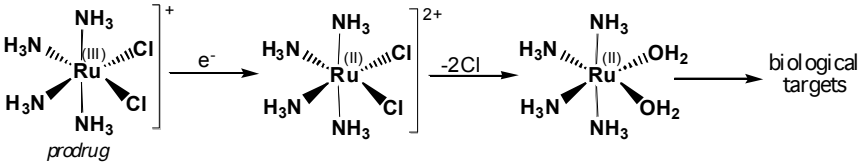
Ruthenium complexes are presently a subject of great interest in the field of biological, pharmaceutical and medicinal chemistry. In the past, they have been widely investigated as immunosuppressants, nitric oxide scavengers, antimicrobial agents or antimalarial agents. But most of all, ruthenium complexes have attracted interest for their potential use as anti-cancer agents in the past 30 years. In detail, ruthenium compounds show low systemic toxicity and appear to penetrate well the tumor cells, to bind effec-

tively DNA and proteins and to present, in some cases, selective antimetastatic properties.

The capacity of such compounds to bind to imine groups with a relatively high affinity has raised a great interest about the possibility to develop anticancer agents. It is known, in fact, that imine groups are present both in proteins (histidine residues) and in nucleic acids.

Ruthenium complexes have peculiar properties with respect to Pt(II) drugs. For example, two different oxidation states are accessible for the ruthenium ion in aqueous solution: Ru(II) (d6, diamagnetic) and Ru(III) (d5, paramagnetic). Therefore, the ruthenium complexes are redox-active agents that can undergo electron-transfer reactions with biological reactants (e.g. gluthatione). Due to their octahedral geometry, ruthenium compounds most likely function in a different manner than the square-planar platinum anticancer agents. Finally, the ability of ruthenium to mimic iron in binding to many biological molecules, including serum proteins (e.g. transferring and albumin) is believed to contribute to the general low toxicity of ruthenium drugs. However, the kinetics of ligand exchange for ruthenium complexes are similar to those of Pt(II)compounds. Also, similarly to platinum, ruthenium is endowed with high affinity for nitrogen and sulphur ligands.

In 1980 Clarke and co-workers suggested that inert Ru(III) complexes may serve as prodrugs activated in vivo by reduction to more labile Ru(II) species. These latter species can coordinate to biomolecules after hydrolysis of some ligands.56 (Schema1.1) Glutathione and a number of redox proteins are indeed capable of reducing [RuCl(NH3)5]2+ in vivo.



Scheme 1.1

In 1984 Mestroni and co-workers studied the antineoplastic activity of a ruthenium(II) complex, [cis,fac-RuCl2(dmso-S)3(dmso-O)] in comparison with that of cisplatin, using three metastazing mice tumors: Lewis lung carcinoma, B16 melanoma, and MCa mammary carcinoma. [cis,fac-$RuCl_2$(dmso-S)$_3$(dmso-O)] was found to interact in vitro with DNA forming covalent bonds with the nucleobases, especially guanine (N7), even though to a considerably lower extent compared to cisplatin.

In 1986 the group of Keppler investigated the antitumor activity of Ru(III) complex [ImH][trans-$RuCl_4$(Im)$_4$] (ICR, where Im=imidazole) in the P388 leukemia, the Walker 256 carcinosarcoma, and the intramuscularly transplanted sarcoma 180. ICR is considered the progenitor of a new generation of ruthenium-dimethyl sulfoxide complexes and has been the object of a number of chemical and mechanistic studies.

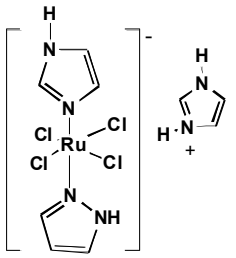


Figure 1.5 Schematic structure of [ImH][trans-$RuCl_4(Im)_4$] (ICR).

In 1988 the Ru(II) complex [trans-$RuCl_2(DMSO)_4$, developed by Alessio and Mestroni, was shown to be more active than the cis isomer [cis,fac-$RuCl_2$(dmso-S)$_3$(dmso-O)] against Lewis lung carcinoma, a matastasizing murine tumor.

In 1992 Alessio and Mestroni reported the antitumor and antimetastatic activities of the Ru(III) compound Na[trans-$RuCl_4$(Im)(DMSO) (NAMI). Since metastases of solid tumors are highly difficult to treat and represent the main reason of failure of cancer therapy this represented an important development.

Later on, NAMI was replaced by its imidazolium analog [ImH] [trans-$RuCl_4$(Im)(DMSO)], NAMI-A, which is endowed of the same antimetastatic properties, but is more soluble, more stable and reproducible solid and consequently is more suitable for further development in clinical phase.

NAMI-A exhibited remarkable results in the preclinical tests and, in October 1999, it entered phase I clinical trials at the Netherlands Cancer Institute of Amsterdam, first compound of ruthenium ever to do so. NAMI-A passed this evaluation after testing in 24 patients showing good tolerability over a wide range of sub-toxic doses without any unexpected toxicity. Although no formal common toxicity criteria (CTC) developed, painful blister formation was considered dose limiting and the advised dose for further testing of NAMI-A was determined to be 300 mg/m^2/day.

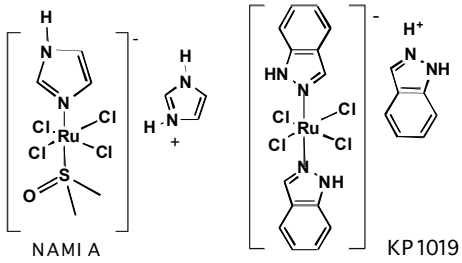


Figure 1.6 Schematic drawings of [ImH] [trans-$RuCl_4$(Im)(DMSO)] (NAMI-A) and [INdH][trans-$RuCl_4(Ind)_2$] (KP1019).

In 2003, another ruthenium(III) compound developed by Keppler and co-workers, [INdH][trans-$RuCl_4(Ind)_2$] (KP1019, where Ind=indazole), active against platinum resistant colorectal tumors in animal models, showed encouraging pharmacological properties and low toxicity and has now entered phase I clinical trials. Significant re-

search on the solution chemistry of KP1019 has influenced on the medical applications of this complex.

The poor water solubility of compound KP1019, makes it difficult to prepare a formulation appropriate for clinical trials. To solve this problem, new complex salts were synthesized by ion exchange of the indazolium cation against a variety of cations, in such a way that the complex anion, responsible for anti-tumor activity, remains unchanged. Especially the sodium salt shows a much better water solubility, while the corresponding tetra-phenylphosphonium salt and the tetraalkylammonium salts are much more soluble in organic solvents, which might be of interest for further reactions with these complex salts.

In preclinical investigation, the activity of KP1019 was observed against non-small cell lung, breast and renal cancers.72 This has inspired considerable interest in the study on the biochemical behaviour of this complex including its interactions with proteins.

More recently, increasing interest has focused on organometallic compounds, specifically a number of ruthenium(II) arene compounds were shown to possess very encouraging cytotoxic and antitumour properties in preclinical models , and are being intensely investigated.

A series of compounds with the general formula [Ru(η6-arene)Cl(en)][PF_6] (en=ethylenediamine; arene=benzene, p-cymene, tetrahydroanthracene, etc.) have been studied for their in vitro anticancer activity; e.g., one of the most active complexes, [Ru(η6-p-cymene)Cl(en)][PF6], exhibits an IC50 value of 8 μM against the A2780 human ovarian cancer cell line.

In the same paper [Ru(η6-p-cymene)Cl_2(isonicotinamide)] was described but found to be much less active than the ethylenediamine complexes.

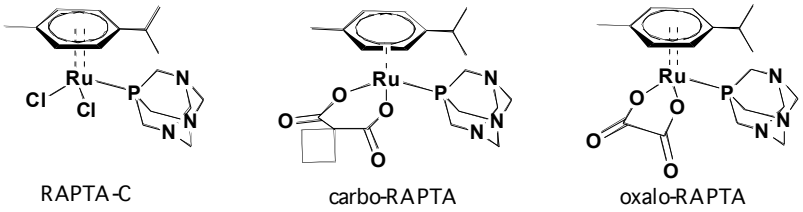


Figure 1.7 Schematic drawing of the selected ruthenium(II)-arene capped complexes.

Of great interest are also the compounds of general formula [Ru(η6-arene)Cl_2(pta)] (pta= 1,3,5-triaza-7-phosphaadamantane) (RAPTA), the prototype being [Ru(η6-p-cymene)Cl_2(pta)] (RAPTA-C). In vitro RAPTA-C exhibits pH-dependent DNA-damaging properties such that at pH < 7, which can be found in the tumor mass of poorly oxygenated cancer cells, DNA is damaged, whereas normal cells with pH > 7 are not affected.

In vivo, RAPTA-C was found to significantly reduce the growth of lung metastases in CBA mice bearing the MCa mammary carcinoma.

Mechanism of actions

The final targets and the mechanisms through which ruthenium complexes exert their antitumor effects are largely unknown, and controversial opinions still exist on this issue.

Ruthenium occurs in aqueous solution mainly as Ru(II) and Ru(III) and both ions are always six-coordinate with octahedral geometry as opposed to the square-planar geometry of Pt(II); these structural facts suggest that ruthenium antitumor complexes probably function in a manner differently that cisplatin, utilizing other and multiple biological mechanisms for transport and macromolecular binding.15 However, there is substantial cytological data that nucleic acids, particularly nuclear DNA, are the target for many ruthenium complexes. In most cases, DNA binding by ruthenium complexes has been associated with anticancer activity.

While these complexes appear to be transported in blood mostly by transferrin (80%) and to a lesser extent by albumin, its localization in tumor has been attributed to this transport mechanism because tumor cells express a large number of transferring receptors on their membranes.

The tumor selectivity of ruthenium compounds is based on two points.

1. Ruthenium(II) ions can be more cytotoxic than the corresponding ruthenium(III) anions. The main conclusion from a long set of physico-chemical investigations was in favour of the possibility of having ruthenium(III) prodrugs which are relatively inactive and unreactive, and devoid of intrinsic cytotoxicity, that can be activated to the corresponding more reactive ruthenium(II) species depending on the redox potential of the tumor environment.

Such compounds, when administered, should behave like non-toxic species until they reach the solid mass of a tumor tissue passing a reducing environment due to the characteristics of the tumor growth. In this environment, with its lower oxygen content and pH than healthy tissue, reduction to the more reactive Ru(II) oxidation state can take place. In that way, ruthenium(III) prodrugs will be more reactive towards biological targets and therefore more toxic for the tumor under consideration.

2. Ruthenium ions have a particular tropism for tumors, higher than for the normal tissues and, moreover, they could be delivered by transferring and concentrated to tissues rich in transferring receptors (i.e. tumor tissues).

There is a significant paper that reports that a tranferrin-ruthenium complex can be transported actively to tumor tissues. It must be stressed that ruthenium anions themselves exhibit high propensity to bind to tumor cells. For some ruthenium complexes studied, the ratios between ruthenium bound to tumor cells and normal tissues, including blood, muscle and liver, are clearly in favour of tumor cells. These ratios were significantly increased for ruthenium chloride when the compound was bound to transferring and injected into mice as a ruthenium-transferrin complex.

The low toxicity of ruthenium drugs is also believed to be due to the ability of ruthenium to mimic iron in binding to many biological molecules, including serum transferrin. These two proteins are used to solubilise and transport iron, thereby reducing its toxicity. Accumulation of ruthenium complexes an tumor tissue is possibly because tumor cells, growing more rapidly than normal cells require a larger amount of iron, and have a high nember of transferring receptors.

1.1.3 Gold complexes as antitumor agents

The serendipitous discovery of the anticancer properties of cisplatin, dating back to 1965, has prompted a great deal of interest in the field of anti-tumour metallodrugs. The very favourable pharmacological profile shown by cisplatin suggested that other metal-based compounds might similarly possess attracting anti-tumour effects while (hopefully) exhibiting a different spectrum of biological activities and a lower systemic toxicity.

In the search of novel anticancer metallodrugs, gold based pharmacological agents have attracted researchers' attention in the last years.

Since d8 Au(III) is isoelectronic and isostructural with Pt(II), square planar gold(III) compounds soon appeared to be excellent and innovative candidates for anticancer testing. However, at variance with platinum(II) compounds, Au(III) analogues were readily found to manifest, on the whole, a rather poor stability profile being kinetically more labile than the corresponding platinum(II) compounds, light-sensitive and easily reducible to metallic gold. As a result of these difficulties and, also, of detection of important in vivo systemic toxicity, gold(III) compounds were quickly abandoned.

Nonetheless, during the 90's, there was a strong return of interest toward gold(III)-based compounds as anticancer agents, especially when a few novel gold(III) compounds exhibiting improved stability, lower toxicity and favourable in vitro pharmacological properties, were prepared and made available for pharmacological testing. For instance, a series of organogold(III) DAMP (DAMP = o-$C_6H_4CH_2NMe_2$) complexes, with formula [Au(DAMP)X_2], where X is a halide, were prepared and characterized by Buckley, Parish and Fricker, and screened for anti-tumour activity, with encouraging results.

Later on, in the attempt of obtaining pharmaceutically useful substances with an even better stability profile, some classical square planar gold(III) complexes, based on a variety of structurally different ligands, were synthesized and characterised in our laboratory.

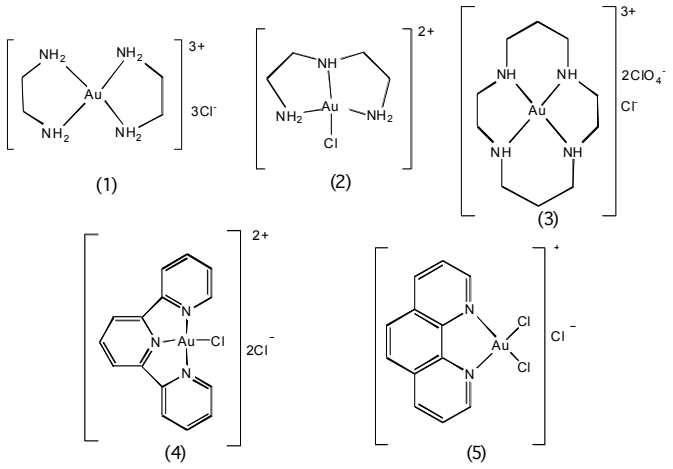


Figure 1.8 Schematic drawing of (1) [Au(en_2)]Cl_3, (2) [Au(dien)Cl]Cl_2, (3) [Au(cyclam)]$(ClO_4)_2Cl$, (4) [Au(terpy)Cl]Cl_2, and (5) [Au(phen)Cl_2]Cl.

To increase the stability of the gold(III) center, multidentate ligands such as polyamines, cyclam, terpyridine and phenathroline were preferentially employed. A few compounds - namely [Au(en2)]Cl3, [Au(dien)Cl]Cl_2, [Au(cyclam)]$(ClO_4)_2Cl$, [Au(terpy)Cl]Cl2, and [Au(phen)Cl_2]Cl^- - were characterised both in the solid state and in solution (Figure 1.8).

Their solution behaviour was analysed in depth through the application of various physico-chemical methods such as visible absorption spectroscopy, ESI mass spectrometry, and chloride-selective potentiometric measurements.

Overall, a quite satisfactory stability profile in buffered aqueous solutions emerged for all these classical gold(III) compounds that opened the way to their in vitro pharmacological testing. Their cytotoxic properties were primarily assessed by the sulforhodamine B assay on the representative human ovarian tumour cell line A2780, either sensitive (A2780/S) or resistant (A2780/R) to cisplatin. In most cases, the mentioned compounds revealed important in vitro cell killing properties, with IC50 values generally falling in the low micromolar range; additionally, these compounds turned out to overcome- to a large extent- resistance to cisplatin in the cisplatin-resistant cell line making them particularly attractive for further pharmacological evaluation. Some relevant cytotoxicity data are reported in Table 1.

In the same years, the solution behaviour and the cytotoxic properties of hyphenate chloro- glycylhistidinate gold(III) GHAu), a complex with promising chemical and biological properties, was reported. Notably, this gold(III) peptide complex manifested a far higher cytotoxic activity towards the established A2780 ovarian carcinoma human cell line (see Table 1) compared to its zinc(II), palladium(II), platinum(II) and cobalt(II) analogues, proving that the gold(III) centre has a crucial role in determining the pharmacological effects.

Table 1.1 Cytotoxicity (IC50 μM) of the gold compounds studied in Florence during the last years towards different tumour cell lines. Cisplatin is reported as reference compound. Data were collected after 72 h exposure to drug.

Compounds	A2780/S	A2780/R	CCRF-CEM/S	CCRF-CEM/R	SK-OV-3	MCF7	HT29	A549
cisplatin	1.2±0.43	14±2.72	0.7±0.1	20.1±7.2	5.2	5.30±0.87	6.30±0.23	-
[Au(en)$_2$] Cl$_3$	8.36±0.77	17.0±4.24	-	-	-	-	-	-
[Au(dien)Cl] Cl$_2$	8.2±0.93	18.7±2.16	12.6±2.0	32.7±6.6	-	-	-	-
[Au(cyclam)]ClO$_4$)$_2$Cl	99.0	>120.0	-	-	-	-	-	-
[Au(Terpy)Cl]Cl$_2$	0.2	0.37±0.032	-	-	-	-	-	-
[Au(Phen)Cl$_2$]Cl	3.8±1.1	3.49±0.91	2.3	6	-	-	-	-
[Au(bipy^{c}-H)(OH)] [PF$_6$]	3.3±1.4	8.2±1.5	11.9±2.1	51.2±5.6	13.3±1.6	35.30±8.8	24.60	>50
[Au(bipy)(OH)$_2$] [PF$_6$]	8.8±3.9	24.1±8.7	52.9±11.6	58.6±0.9	34.4±4.7			
Au(bipy^{dmb}-H)(2.6-xylidine-H)][PF$_6$]	2.50±0.43	5.7±0.3	-	-	-	5.20±0.40	~25	~35
Au(py^{dmb}-H)(AcO)$_2$]	2.90±0.34	6.40±1.0	-	-	-	17.70±0.44	8.60	~49
Auoxo1	22.8±1.53	23.3±0.35	-	-	-	-	-	-
Auoxo2	12.1±1.5	13.5±1.8	-	-	-	-	-	-
Auoxo3	25.4±2.47	29.8±3.1	-	-	-	-	-	-
Auoxo4	12.7±1.06	19.8±1.8	-	-	-	-	-	-
Auoxo5	11.0±1.5	13.2±1.2	-	-	-	-	-	-
Auoxo6	1.79±0.17	4.81±0.5	-	-	-	-	-	-

A novel series of gold(III) compounds, of potential use as anticancer agents, namely the gold(III) phenylpyridine (ppy) derivatives were described by Fan and Ranford at the beginning of the 2000's. [Au(ppy)Cl_2], forming a five membered cycloaurated chelated ring with the bidentate ppy ligand, is the reference compound for this family. The two remaining coordination positions in [Au(ppy)Cl_2] are occupied by two chloride groups in cis to each other, thus conferring some structural analogy to cisplatin. Inspired by [Au(ppy)Cl_2], Fan and Ranford prepared and characterised a number of analogues containing carboxylate ligands in the place of chloride to improve aqueous solubility. The main structural aspects of these complexes was unambiguously elucidated by X-ray diffraction studies. All these gold(III) complexes were then tested for cytotoxic properties in vitro against MOLT- 4 (human leukemia) and C2C12 (mouse tumour) cell lines.

A cytotoxicity profile similar to cisplatin was found in the case of MOLT-4 cell whereas no significant activity was observed on the C2C12 cell line.

In 2002, in collaboration with the group of Minghetti and Cinellu (University of Sassari, Italy), we proposed that a few novel gold(III) complexes, bearing the bipyridyl motif (bipy), might be pairwise assayed for antiproliferative effects . This family of compounds turned out to be acceptably stable within a physiological buffer and to induce outstanding tumour cell growth inhibition. In particular, our investigations focused on two members of this class, namely [Au(bipyc-H)(OH)][PF6] and [Au(bipy)(OH)2][PF6] (Figure 1.9).118 A number of analogues were subsequently prepared and characterized (see Figure 1.9, compounds 3 and 4).

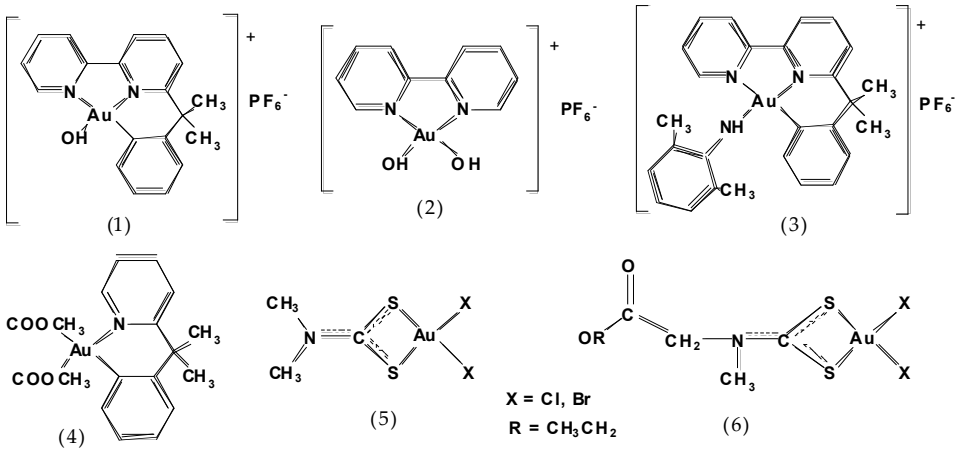


Figure 1.9 Schematic drawing of [Au(bipyc-H)(OH)][PF6] (1), [Au(bipy)$(OH)_2$][PF_6] (2), Au(bipydmb-H)(2.6-xylidine-H)][PF_6] (3), Au(pydmb-H)$(AcO)_2$] (4) (where pydmb = 2-(1,1-dimethylbenzyl)-pyridine)) and of the gold(III) dithiocarbamate complexes containing N,N-dimethyldithiocarbamate (5) and ethylsarcosinedithiocarbamate (6) ligands.

In [Au(bipy)$(OH)_2$][PF_6], two adjacent positions of the square planar gold(III) chromophore are occupied by two nitrogen atoms from the bipyridyl ligand while hydroxide groups are the ligands at the two remaining coordination positions. At variance, [Au(bipyc-H)(OH)][PF_6] (where bipyc = 6-(1,1-dimethylbenzyl)-2,2'-bipyridine) is an organogold(III) complex in which donors to the gold(III) center are two nitrogens from the bipyridyl ligand, the C2 carbon of the phenyl group, and a hydroxide group. Only small deviations from ideal square-planar geometry were observed in the classical bipyridyl complexes whereas such deviations are quite large in the case of cyclometallated derivatives due to a limited flexibility of the N,N,C-ligand. These compounds are sufficiently soluble in aqueous buffers. Their intense visible bands lying in the 300-370 nm region, LMCT in nature and diagnostic of gold oxidation state +3, were exploited to monitor reactivity with various kinds of biomolecular targets. Notably, these two complexes manifested a clearly different behavior in their reactions with ascorbate as only the [Au(bipy)(OH)2]+ species was shown to undergo reduction. This means that the oxidation state +3 is far more stable in the organogold(III) species compared to [Au(bipy)$(OH)_2$][PF_6], in line with previous electrochemical results.

The in vitro cytotoxic properties of these bipyridyl gold(III) complexes were determined toward the human ovarian carcinoma cell line A2780, either sensitive or resistant to cisplatin. Both gold(III) complexes showed important cell killing effects, with IC50 values again falling in the low micromolar range (see Table 1). [Au(bipyc-H)(OH)][PF_6] resulted to be the most active with at least a twofold higher activity than cisplatin in the A2780/R cell line. The cytotoxic properties of these complexes were also evaluated on the human ovarian cell line SKOV3 (inherently resistant to cisplatin) and on the CCRF-CEM leukemic cell line, either sensitive (CCRF-CEM/S) or resistant (CCRF-CEM/R) to cisplatin. When assayed on these cell lines, both gold(III) complexes were generally less active than on the A2780 line. In any case, these gold(III) compounds retained, to a large extent, their activity toward the cisplatin-resistant A2780/R and CCRF-CEM/R lines suggesting that the mechanisms of resistance to cisplatin -most likely intracellular detoxification and increased repair of DNA damage- are scarcely effective toward these gold(III) complexes. In addition, preliminary studies have pointed out quite unambiguously that the interactions of these complexes with DNA are weak whereas relatively tight adducts are formed in the reactions with model proteins and serum proteins .

In the same years, Fregona and coworkers prepared and characterised a few novel gold(III) dithiocarbammate compounds showing a very encouraging biological profile. The compounds containing N,N-dimethyldithiocarbamate and ethylsarcosinedithiocarbamate ligands (compounds 5 and 6 in Figure 1.9) were the most intensely studied members of this family. Early in vivo data on murine models were very promising and led to the patenting of these novel compounds. Afterwards, several additional investigations were carried out aimed at elucidating some aspects of their mechanism of action, both at the cellular and biochemical level. It is worthwhile mentioning that very recent results strongly suggest that these gold(III) dithiocarbammate compounds act though an unexpected and unprecedented mechanism, i.e. inhibition of the cancer cell proteasome.

Concomitantly, a series of interesting gold(III) meso-tetraarylporphyrins (TPP) complexes were described by Chi Ming Che, Hongzhe Sun and co-workers at the University of Hong Kong. These compounds, of general formula [Au(III)(p-Y-TPP)]Cl, [with Y = H, Me, OMe, Br and Cl], were characterised by classical physiochemical methods. Notably, coordination to the tetrapyrrole ring results into a large stabilization of the gold(III) oxidation state. Accordingly, these gold(III) porphyrins showed a high stability in aqueous solutions around neutral pH as well as an appreciable stability against glutathione (GSH), a biological reducing agent commonly present, at millimolar concentrations, inside cells.

All the mentioned gold(III) porphyrins revealed large in vitro antiproliferative effects, with IC50 ~ 0.1–1.5 μM, thus being significantly more effective that cisplatin itself. The lack of cross resistance with classical platinum(II) compounds again suggests that gold(III) porphyrins and cisplatin induce cytotoxicity through distinct mechanisms. Remarkably, zinc(II) porphyrin [Zn(II)(TPP)] was comparatively investigated and found to be at least 100-fold less cytotoxic than gold(III) porphyrins (IC50 > 50 μM), thus highlighting the crucial role of the gold(III) centre in causing the biological actions. Nonetheless, the porphyrin ligand was found to be essential for the anticancer activities leading these authors to conclude that the porphyrin ligand is crucial both in stabilizing the Au(III) center and in delivering the metal to its cellular targets.

DNA is one of the major putative targets for metal-based anticancer drugs. Thus reactions of gold(III) porphyrins to DNA were studied extensively. In particular, Hongzhe Sun and coworkers examined the interactions of representative gold(III) porphyrins with duplex DNA by UV-vis absorption titrations. Isosbestic changes and significant hypochromicity of the Soret band were noticed in the electronic spectra of gold(III) porphyrins after addition of calf thymus DNA suggesting a direct and tight interaction with the DNA double helix. Moreover, by using confocal microscopy, these gold(III) porphyrins were shown to induce extensive apoptosis in HeLa cancer cells. In a subsequent study the same authors reported that treatment with gold(III) porphyrins induces significant and characteristic changes in protein expression profiles.

A quite common strategy in the field of anticancer metallodrugs has been the design and the preparation of dimetallic or polymetallic compounds, derived from the "fusion" of two or more monometallic molecular fragments, in which the specific reactivity of each metal centre is further controlled by its interactions with the nearby metal centre(s) and by the overall molecular framework. Notably, incorporation of two (or more) metal centres within an extended molecular framework may greatly affect the overall charge of the resulting polynuclear compound, its redox properties, the kinetics of hydrolysis, and its specific reactivity toward biomolecules in comparison to mononuclear analogues. Notable examples of this strategy have concerned platinum(II) and ruthenium(III) anticancer metallodrugs.

These observations led us to prepare novel dinuclear gold(III) compounds to be tested as anticancer agents, starting from the mononuclear gold(III) bipyridyl complexes described above.118 A series of six dinuclear gold(III) oxo complexes with bipyridyl ligands (Figure 1.10), of general formula $[Au_2(N,N)2(\mu\text{-}O)_2][PF_6]_2$ [where N,N = 2,2'-bipyridine (Auoxo1), 4,4'-di-tert-butyl- (Auoxo2), 6-methyl- (Auoxo3), 6-neopentyl- (Auoxo4), 6-(2,6-dimethylphenyl)- (Auoxo5), 6,6'-dimethyl-2,2'-

bipyridine (Auoxo6)], were thus prepared and characterised in our laboratory, and their antiproliferative properties evaluated in vitro toward the reference A2780 human ovarian carcinoma cell line (see Table 1).

While five compounds of this series manifested only moderate cytotoxic properties (with IC50 typically in the 10-30 micromolar range), the sixth one (Auoxo6), turned out to be ~5-15 times more active against both cell lines, thus meriting further investigations. In particular, much attention was focused on the chemical and structural reasons for the higher biological activity of Auoxo6. Remarkably, a rather evident positive correlation emerged in subsequent studies between the oxidising power of this compound, its reactivity with biomolecular targets and its antiproliferative effects.

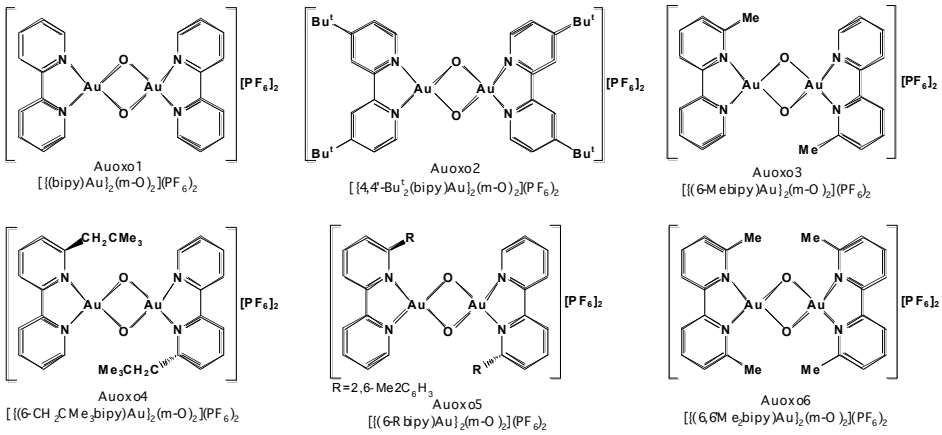


Figure 1.10 Schematic drawings of the dinuclear gold(III) complexes Auoxo. Auoxo3 is a ca. 1:1 mixture of the cis and trans isomer while Auoxo4 and Auoxo5 are, as depicted, only trans isomers.

The mechanism of action: some insights

As outlined above, the renaissance of interest toward gold(III) compounds as potential anticancer metallodrugs has resulted, in the course of the last decade, in the obtainment of a conspicuous number of structurally diverse gold(III) species, endowed with sufficient chemical stability and relevant biological activities. Detection of in vitro cytotoxicity has represented, for these metal-based agents, the primary screening criterion in order to assess their potential anticancer properties. IC50 values of 10-5 M or lower were taken as an index of a promising or at least acceptable anti-tumour efficacy in vitro.

Nonetheless, for a few of these compounds, the pharmacological studies were extended well beyond assessment of mere in vitro cytotoxicity by analysing some of the effects they produce at the cellular level such as direct DNA damage, modification of the cell cycle, alterations of mitochondrial functions, induction of apoptosis and so on. Moreover, the reactions of some cytotoxic gold(III) complexes with some specific biomolecular targets, e.g. calf-thymus DNA and a few representative proteins, were

analysed at the molecular level by taking advantage of a vast array of biochemical, biophysical and physico-chemical methods.

On the whole, these cellular and biochemical studies have provided further and valuable insight into the reactivity and the mode of functioning of novel gold(III)-based metallodrugs. Although the ultimate molecular mechanisms of these new anticancer agents still remain largely unknown, some considerations and suggestions may be anticipated based on the available information.

Most of the mechanistic studies carried out on cytotoxic gold(III) compounds have generally been referred and compared to the behaviour of cisplatin; however, it clearly emerges from the experimental results collected so far that the respective molecular mechanisms are rather distinct. In fact, the few gold(III) compounds on which advanced pharmacological testing was performed offer a rather variegate mechanistic profile that drastically contrasts the quite homogeneous biological patterns provided by classical platinum(II) compounds. Only in few cases –e.g. gold(III) porphyrins- some solid evidence for direct DNA damage has been obtained; however, for most other cases, the effects on DNA and on the cell cycle appear to be very modest so that it is very unlikely that DNA may be the "true" primary target.

For instance, it is shown that the large cytotoxic and proapoptotic effects induced by the gold(III) complexes $[Au(phen)Cl_2]Cl$ and $[Au(dien)Cl]Cl_2$ just result into a very marginal DNA damage; accordingly only weak cell cycle effects were detected, at variance with cisplatin (that is known to produce an evident G2/M arrest), further suggesting a modest impact on DNA related functions. A similar situation was previously reported for gold(III) DAMP compounds similarly showing very weak DNA interaction properties.111 Thus, DNA is likely not to be the primary target for several of the novel gold(III) compounds. Even in the cases where some evidence of a direct interaction with DNA has been achieved, the mechanisms of DNA damage and cell death appear to be profoundly distinct from those induced by platinum drugs. All these arguments suggest that alternative biochemical mechanisms must be operative, most likely associated to selective modification of some crucial proteins. To this respect it is worthy reminding that gold(I) and gold(III) compounds are known to target, rather strongly and selectively, thiol groups of proteins (as well as selenol groups); this type of reactivity might reasonably represent the true molecular basis for their biological actions.

In this regard, a recent theory postulates that gold compounds exert their relevant cytotoxic and proapoptotic actions through direct antimitochondrial effects: this idea originally developed for classical antiarthritic and cytotoxic gold(I) compounds like auranofin and aurothiomalate (nonetheless showing a relevant cytotoxic activity in vitro) has later been extended to novel anticancer gold(III) complexes. According to this theory the clue event for the biological mechanism of gold compounds is a direct, strong inhibition of thioredoxin reductase (TrxR), a selenoenzyme critically involved in the regulation of the intracellular redox state and of the mitochondrial functions. As a consequence of large TrxR inhibition the opening of the mitochondrial pore is induced, eventually leading to cyt c release and to the triggering of apoptosis.

Recent studies have shed light into the molecular and structural aspects of thioredoxin reductases. The thioredoxin reductases are enzymes belonging to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases that also includes

lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase. Members of this family are homodimeric proteins in which each monomer includes an FAD prosthetic group, an NADPH binding site and an active site containing a redox-active selenol group. Electrons are transferred from NADPH via FAD to the active-site selenol of TrxR, which then reduces the substrate. TrxRs are named for their ability to reduce oxidized thioredoxins (Trxs), a group of small (10±12 kDa) ubiquitous redox-active peptides which have a conserved -Trp-Cys-Gly-Pro-Cys-Lys- catalytic site that undergoes reversible oxidation/reduction of the two Cys residues.

The crystal structure of thioredoxin reductase has been recently solved . Notably, these structural studies evidenced the presence of a selenocysteine group at its active site that, in the reduced form, displays a high reactivity toward "soft" metal ions. There is today sufficient evidence that this selenol group is the primary anchoring site for a vast array of metals and metal complexes that are known to inhibit thioredoxin reuctase.

Table 1.2 Inhibition (IC_{50} μM) of Thioredoxin reductase by gold compounds. Cisplatin is reported as reference compound.

Compound	IC_{50} (μM)		
	Rat Mitochondrial TrxR2	Rat Cytosolic TrxR1	Human Cytosolic TrxR1
Cisplatin	36.0		
Auranofin	0.020		
Au triethylphosphine chloride	0.065		
aurothiomalate	0.280	0.005	
[Au(2,2²-diethylendiamine) Cl]Cl_2	0.420		0.20
[(Au(2-(1,1-dimethylbenzyl)-pyridine) $(CH_3COO)_2$]	1.420		
[Au(6-(1,1-dimethylbenzyl)-2,2²-bipyridine)(OH)] (PF_6)	0.280		
[Au(6-(1,1-dimethylbenzyl)-2,2²-bipyridine)-H) (2,6-xylidine)] (PF_6)	0.210		
Tetrachloroaurate			0.0058
a			0.012
b			0.030
c			0.036
d			0.180
e			0.030
f			0.0022
g			1.80
h			0.68
Au triphenylphosphine chloride		0.001	
Au(III) chloride trihydrate		0.00075	
Au acetate		0.040	
Au thiosulfate Na^+		0.0005	

Thus, it is straightforward to propose that gold(III) compounds exert their cytotoxic effects by causing direct mitochondrial damage through selective modification of the active site selenol in thioredoxin reductase as previously suggested for auranofin and analogues. As a matter of fact, in a recent study conducted in collaboration with the

group of Bindoli and Rigobello in Padova, we observed that a few gold(III) complexes developed in our laboratory behave as tight inhibitors of the cytosolic form of the selenoenzyme thioredoxin reductase (TrxR) (see Table 1.2), Accordingly, these compounds were found to perturb greatly the mithocondrial functions.

This view is now reinforced by a recent paper by Powis et al. reporting on the inhibitory properties of a series of gold(III) complexes (Figure 1.11), against human thioredoxin reductase 1 (TrxR1), including some gold(III) DAMP compounds. Table 1.2 contains a compilation of representative literature data for the inhibition of TrxR by various gold compounds.

Thus, according to the above reported data, it is reasonable to propose that direct antimitochondrial effects are the determinant for the large proapoptotic and cytotoxic effects produced by anticancer gold(III) compounds.

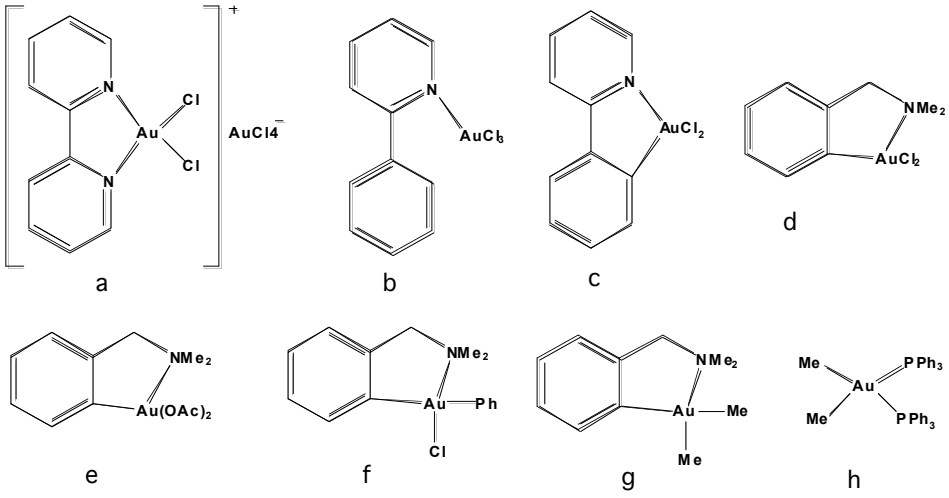


Figure 1.11 Schematic drawings of gold(III) complexes screened for the inhibition of human TrxR1.

Nonetheless, other recent studies identified the thiol-dependent cathepsin enzymes as possible, alternative targets for gold-based anticancer agents. These lysosomal enzymes are mainly cysteine proteases responsible for extracellular matrix degradation, bone resorption and joint destruction. Their marked inhibition arising from coordination of gold complexes to the active site cysteine has recently been reported.

1.2 Metal complexes in the treatment of rheumatoid arthritis

Rheumatoid arthritis is an inflammatory disease of unknown origin characterized by a progressive erosion of the joints resulting in deformities, immobility and a great deal of pain. It is an autoimmune disease in which the body's immune system mounts a re-

sponse against itself. This results in a malign growth of the synovial cells (the cells lining the joint) called a pannus, and infiltration of the joint space by cells of the immune system, primarily macrophages, and associated production of immunoglobulin proteins called rheumatoid factors. Phagocytic cells release degradative enzymes such as collagenase, and generate reactive oxygen species OH and $O2^{-}$, all of which contribute to the resulting tissue damage. This progressive inflammatory response is promoted by raised levels of chemical mediators such as prostaglandins, leukotrienes and cytokines.

This disease affects more than 1% of the general population in the developed countries. No curative therapy exists at present. Medical treatments rely both on first-line symptomatic drugs, such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAID), and second-line therapeutic agents, commonly referred to as disease-modifying arthritis rheumatoid drugs (DMARD). Remarkably, the latter offer the potential for a suppression of the inflammatory activity and for a reduction of the disease progress.

It is surprising that active rheumatoid arthritis can be brought to remission by treatment with metal compounds such as gold or copper complexes or with metal-complexing agents such as penicillamine or 5-aminosalicylate. In some way, the remission-inducing agents must interfere with crucial mechanisms underlying the chronicity of the disease. Recent research indicates that activated tissue macrophages and blood monocytes invading the synovial tissue play a central role in the early steps of pathogenesis and chronification of rheumatoid arthritis. Important signal substances derived from the activated macrophages are the free oxygen radicals (superoxide and hydrogen peroxide) and the cytokines such as tumour necrosis factor-a (TNF-a).

Apparently, these mediating substances play key roles in the progression of the rheumatoid inflammation. Another possible source of free oxygen radicals is related to the anoxic reperfusion reactions that may accompany excessive motions of affected joints. Metal compounds and chelators are presumed to interact with the generation or toxicity of activated oxygen species.

1.2.1 Gold compounds

Gold(I) compounds are well known DMARD agents that were discovered empirically and have been employed in the clinics since the late 1920s. Antiarthritic gold(I) compounds manifest chemical and structural features that are typical of gold(I) complexes. Generally, gold(I) centers show a strong preference for soft ligands such a phosphines and thiols, and for a linear coordination of two ligands. They comprise the gold(I) thiolates that are given intravenously: sodium aurothiomalate (myochrysine), sodium aurobisthiosulfate (sanochrysine), aurothioglucose(solganol), sodium aurothiopropanol sulfonate (allochrysine), and a more recent oral agent, auranofin, introduced in the clinics in the late 1970s. Thus, for a longe time, gold(I) thiolates have been the principal compounds used in chrysotherapy, the treatment of rheumatoid arthritis with gold-based drugs. In more recent time, the introduction of auranofin promised a safer treatment; however, careful investigations of several clinical trials have pointed out that auranofin is somewhat less effective than the injecatble thiolates. The response of

patients to gold therapy is slow and can usually be appreciated only after 3-6 months. One accepted effect of chrisotherapy is the anti-inflammatory action, but this, alone, cannot account for its effectiveness. Additional mechanisms of action which may contribute to the efficacy of chrysoterapy are antimicrobial activity, reduction of humoral immunity, inhibition of the complement pathway, effects on lymphocytes, monocytes, and neutrophils (especially inhibition of T-cell proliferation and of polymorphonuclear monocyte activation), and enzyme inhibition. Several mechanism may operate in parallel; however, non has been recognized yet as the primary biological action.

In 1985 a new compound, a second-generation drug, auranofin, triethyl-phosphine gold(I) tetraacetatothioglucose, was introduced as an orally bioavailable gold drug for arthritis.

Auranofin is a monomeric lipophilic complex containing linear twofold coordinated gold(I) centers bonded to a tetraacetylthioglucose and a triethylphosphine ligand. It has been structurally characterized by X-ray crystallography in contrast with many of the other gold-based drugs. Auranofin has several advantages over previous gold drugs, not least that it can be taken orally. In fact, being lipophilic, auranofin can be administered orally, in doses of 3–6mg per day, so that this drug has a considerable advantage in this respect over the previous. The likely role of the phosphine ligand in auranofin is to enhance the lipid solubility of the drug to facilitate its absorption in the gut. The different solubility profile of auranofin results in a different biodistribution of gold throughout the body compared with that of the conventional water-soluble drugs, which are injected immediately into the blood stream. So, serum gold levels are reduced and maintained for longer, and there is less retention of gold in the tissues and hence renal toxicity is significantly reduced. These advantages are, however, offset by a reduction in efficacy compared with the oligomeric gold(I) thiolates.

The mechanism of action of the antiarthritic gold compounds is unclear, in part due to the lack of understanding of rheumatoid arthritis. An appreciation of the chemical interaction of gold complexes with naturally occurring ligands in a biological environment is helpful in our understanding of the pharmacological activity of the gold drugs.

1.2.2 Other metal complexes

Low selenium levels have previously been reported in blood plasma and cells from patients with rheumatoid arthritis. The most important biological function of selenium is attributed to its presence in the enzyme glutathione peroxidase, which is a crucial factor in the cellular defence against toxic free radicals. Although oxygen radical formation may be of significance in the pathogenesis of rheumatoid arthritis, no significant clinical improvement was obtained when using nutritionally adequate or moderate doses of selenium supplementation.

Forestier was among the first to report that a copper complex, Cupralene, was effective in the treatment of rheumatoid arthritis. Based on open studies, he concluded in 1949 that copper salts are effective in the treatment of rheumatoid arthritis. They give better results than gold salts in the early stages of the disease, but in cases of longer

standing, they must be used if there is gold intolerance or gold resistance, but whenever gold salts are tolerated they are to be preferred.

These positive results with copper complexes were supported by the studies of other workers. , Hangarter and Lubke167 treated more than 600 patients suffering from rheumatoid arthritis with copper salicylate and reported that 65% became symptom free, 23% improved and 12% of the patients remained unchanged. No serious toxic

disturbances were recorded in association with the treatment. Their studies were not controlled, however, and their reports are difficult to evaluate.

High doses of zinc salts led to significant improvements in symptoms of rheumatoid arthritis in a clinical trial, but controversial results have been reported. When reaching into the intracellular space, zinc is a potent inductor of metallothionine, which is a protein tying up both copper and zinc, and which is also reported to act as an oxygen radical scavenger in biological systems.

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Chapter 2

Metallodrugs/protein interactions

2.The importance of proteins in the overall mechanism of action of anticancer metallodrugs

Since the discovery of the antitumor activity of (cis-$[PtCl_2(NH_3)_2]$) cisplatin, during the 1960's, metal based drugs have been playing a major role in anticancer chemotherapeutic strategies. As a matter of fact, a few platinum compounds are today among the most widely used anticancer agents in the clinics as detailed above. Research in the field is still very active and has been expanded in recent years to include a conspicuous number of non-platinum metallodrugs.

During the last three decades, the interest of the scientific community working on anticancer metal compounds has mostly focused on their interactions with DNA, the commonly accepted "primary" target for platinum compounds, that were described and analysed in hundreds of papers. In contrast, rather surprisingly, the reactions of platinum and non-platinum anticancer metallodrugs with proteins have received very little attention. Only a few biophysical studies have indeed appeared dealing with the interactions of anticancer metallodrugs with proteins. These studies mostly concerned the two major serum proteins albumin and transferrin, as well as metallothioneins, small, cysteine-rich intracellular proteins, primarily involved in soft metal ions storage and detoxification. However, additional studies were carried out also on a few model proteins such as ubiquitin, haemoglobin, myoglobin, cytochrome c and glutathione-S-transferase.

The most relevant achievements obtained in this field until 2005 were excellently summarised by Keppler and coworkers in a comprehensive review appeared on Chem. Rev.

This topics deserves more and more attention as it is increasingly evident that the interactions of anticancer metallodrugs with proteins play crucial roles not only in their uptake and biodistribution processes but also in determining their overall toxicity profile. Even more interesting, reactions of anticancer metallodrugs with proteins are likely to be involved in some crucial aspects of their mechanism of action. This latter statement is particularly true for non-platinum anticancer metallodrugs such as ruthenium and gold compounds for which DNA-independent mechanisms of action have been proposed and experimentally supported. For instance, it was suggested that dimethylsulfoxide ruthenium(III) drugs might either interfere with specific proteins involved in signal transduction pathways or alter cell adhesion processes. Direct antimi-

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tochondrial effects were demonstrated for a few cytotoxic gold complexes with gold in the oxidation states +1 and +3. Thus, further work is absolutely needed to analyse the reactions of metallodrugs with proteins at the molecular level, to identify common trends in these reactions, to characterise the structure and reactivity of the resulting adducts and to identify the most important intracellular protein targets for the various classes of anticancer metallodrugs.

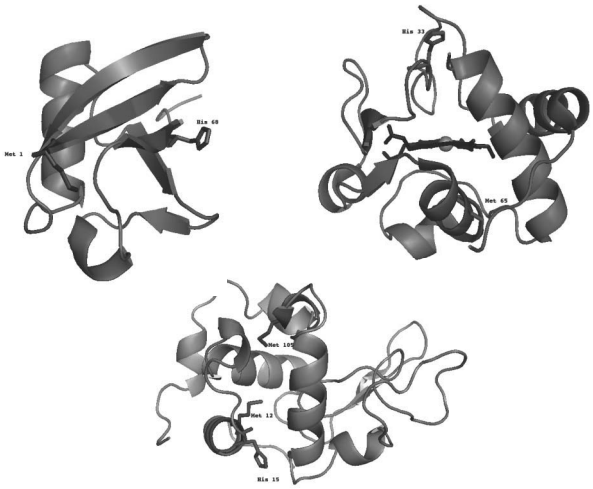

Figure 2.1 Molecular structures of ubiquitin (a), cytochrome c (b) and HEWL (c), with the aminoacidic residues, possible metal binding sites, highlighted as stick graphics. The PDB files are available at the website www.rcsb.org. The figure was generated using Pymol (DeLano Scientific LLC; http//pymol.sourceforge.net).

As it emerges from the review paper by Kratz, early work on the interactions of antitumor metal complexes with plasma components, in most cases transport proteins, has been conventionally performed using various spectroscopic techniques, including electronic, vibrational, circular dichroism (CD), fluorescence, and NMR spectroscopy. Although providing valuable information on the nature and number of protein active sites participating in binding, as well as on its rate, specificity, and reversibility, these techniques require the separation of excess metal from its protein-bound form (typically achieved by ultrafiltration, dialysis, or gel filtration), which makes the whole procedure laborious, time-consuming, and possibly entailing a certain loss of binding. The inadequacy of classical instrumental methods is a consequence of their lack of sensitivity.

Application of more sensitive atomic absorption spectrometry (AAS) and atomic emission spectroscopy (AES) techniques imposes the removal of unbound drug from the incubation solution prior to the metal determination step, which might also be error-prone. Largely as a consequence of these drawbacks, the exact role that binding to proteins plays in the mechanism of the drug's action remains somewhat unclear. In addition, there still exists controversy between the results of different studies regarding

the binding stoichiometry and kinetics, degree of modification of the protein conformation and functions, etc. Last but not least of all, only a few of the aforementioned techniques have been refined to the analysis of metallodrug-protein binding under real-world conditions in blood plasma, however, with a shortcoming of limited selectivity.

Nowadays, more so than in past decades, there is strong concern within the anticancer research community regarding improvement of the arsenal of the analytical techniques in use. This led in particular to a growing number of metallodrug-protein investigations performed using valuable techniques superior to contemporary methods used in the field. For instance, a number of papers have highlighted the great potential of modern mass spectrometry ionization methods, in particular ESI and MALDI MS, to characterise metal-protein adducts at the molecular level. On the other hand, X-ray diffraction studies of such adducts, although not easy, may result extremely valuable in providing detailed structural information on the formed metallodrug-protein species. On the whole, these methods have the potential to offer rather exhaustive descriptions of metallodrug/protein interactions when working on the purified components. Conversely, the rapid development of modern proteomic technologies and the use of advanced protein separation techniques, coupled to very sensitive metal detection methods, hold promise for the successful analysis of complex mixtures of metallated proteins and for the identification of those proteins that act as primary "metallodrug receptors" and/or "metallodrug targets". Thus, these latter techniques open the way to the investigation of far more complicated systems such as metallodrug/treated cell populations and/or cell homogenates, that reflect more closely the reality of metallic species in the cell world.

2.1 Basic aspects of metallodrugs/protein interactions

Most metallodrugs are known to behave as pro-drugs, in other words an activation step is required before they can react with their biomolecular targets and cause their specific biological effects. Usually this step consists of the release of a weak ligand (the so called leaving group) and of its replacement by a water molecule. The resulting aqua species usually manifest a high propensity to react with protein side-chains, showing a pronounced preference for histidine, cysteine and methionine residues, but also for carboxylate groups. Alternatively, activation may take place through a redox process, for instance metal reduction, as it is the case for newly developed anticancer platinum(IV) compounds. The reaction of activated metallodrugs with protein side chains leads to formation of metallodrug-protein complexes or adducts in which metallic fragments are coordinatively bound to proteins. These adducts usually manifest an appreciable stability. However a further reactivity may be expected if the metallic fragments still bears reactive site; if the adduct is reacted with other biomolecules showing a higher affinity for the metal; if the protein possesses stronger, kinetically disfavoured, binding sites for the metallic fragment. Of course, this residual reactivity may be very important in order to assess whether the formed species will conserve some biological activity.

Furthermore, part of the reasons for low productivity of anticancer metallodrug discovery and development is a limited knowledge about the mode, in which the metal complex penetrates the tumor cell and how much is inactivated.

In view of the fact that a vast majority of cytotoxic metal-containing compounds are administered intravenously, special consideration should therefore be given to interactions of the metal drug with macromolecular blood components, which can then be taken up by and accumulated in tumor tissue. In this context, binding toward serum proteins that may perform a transport function for a platinum (or other) metal, for example, albumin or transferrin, appears to be the most important issue, because such interactions determine also the overall drug distribution and excretion and differences in efficacy, activity, and toxicity.

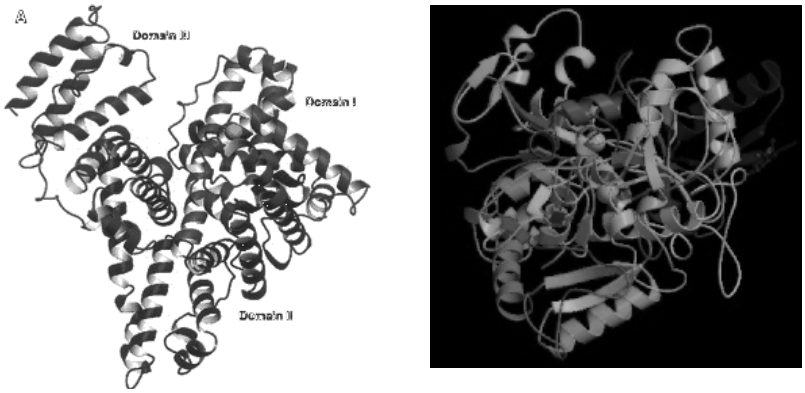

Figure 2.2 Molecular structures of (a) human serum albumin and (b) human serum transferrin.

Human serum albumin (figure 2.2a) is the most abundant plasma protein (about 52%) with a 40-45 g L-1 content in healthy humans (ca. 0.6 mM; Mw 66-67 kDa). It comprises a single chain with 585 amino acids organized in three similar domains (I, II, and III), each consisting of two subdomains (IA, IB, etc.). At physiological pH, albumin adopts helical conformation (67% R-helix content), and its amino acid sequence contains 17 disulfide groups, one thiol group (cysteine-34), and one tryptophan residue (tryptophan-214) with the mutual binding potential toward many types of compounds. The binding sites are located in hydrophobic cavities in subdomains IIA and IIIA. These binding locations were determined crystallographically for several binding partners. The protein was proven to bind and transport a variety of compounds, for example, fatty acids, bilirubin, metal ions, steroid hormones, vitamins, and pharmaceuticals,41,42 including metallodrugs. Serum albumin performs a number of physiologically important functions-control of osmotic blood pressure, transport, metabolism, and distribution of various compounds (including drugs), radical deactivation, and delivery of amino acids after hydrolysis for the synthesis of other proteins.

Human serum transferrin (figure 2.2 b) has a molecular mass of about 80 kDa and is found in blood plasma at a concentration of about 2.5 g L-1 (35 μM). The members of

the transferring group in general possess a high degree of sequence homology, for example, 60% for trans- and lactoferrin. They are single-chain glycoproteins containing ca. 700 amino acids (679 amino acids in the case of transferrin). Transferrin is capable of binding two iron ions in oxidation state +3 (Fe3+ is bound selectively over Fe2+) and acts as an iron transporter.

The iron(III) binding sites are located both in the N- and in the C-terminal lobe, and the two lobes are highly homologous (ca. 40%). Each lobe contains a distorted octahedral Fe3+-binding site consisting of two tyrosines, one histidine, one asparagine, and a bidentate carbonate ion that acts as a synergistic anion in the binding process. Diferric transferrin was found to associate with cells at 37°C more strongly than the monoferric or apotransferrin (in this order of affinity). Once transferrin of whichever type is bound to the receptor it is processed by the cells. Inside the endosomes, the diferric transferrin releases the iron ions due to a lower pH (ca. 5.5) and becomes finally recycled back to the cell surface.

2.2 Model proteins

An essential step in this research area, is represented by the firm characterization and modellisation of the interactions taking place between representative metallodrugs and protein side-chains. It is reasonable to assume that, in spite of the intrinsic structural diversity of the various proteins, some common patterns may hold in metal protein interactions related to the specific nature of the metal center and of protein side-chains. To this scope, studies of simple model proteins have turned out to be valuable. For instance, proteins that were selected in our research group as suitable models to test reactivity with metallodrugs were the following: bovine erythrocyte superoxide dismutase (SOD) (EC 1.15.1.1), hen egg white lysozyme (HEWL) (EC 3.2.1.17), horse heart cytochrome c (cyt c) and bovine erythrocytes ubiquitin (Ub). Schematic drawings of some of these proteins are shown in figure2.1.

For all these proteins high resolution crystal structures are available. Notably these proteins are of moderate to small size with MW ranging from 34000 (SOD in the dimeric form) to 6500 Da. Moreover, all these proteins are commercially available, manifest a high stability in solution under physiological-like conditions, are relatively cheap and water soluble. In addition these proteins exhibit in most cases a basic pI and are thus appropriate for ESI MS detection in the positive mode. All these features render experimental work on these model systems more comfortable.

Superoxide dismutase was selected as the model protein of our study for a number of reasons. The crystal structure of SOD is available at high resolution;48,49 SOD is a medium size protein with several potential binding sites for metals; it is highly water soluble; it is commercially available; it is known to crystallise easily and it exhibits a great stability under physiological-like conditions.

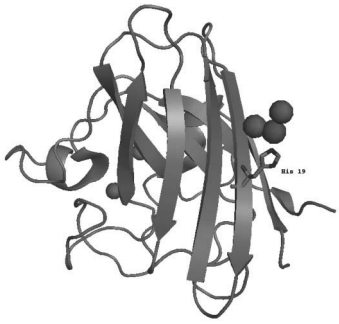

Figure 2.3 Schematic representation of the asymmetric unit containing the physiological monomer of SOD bound to cisplatin; the side chain of His 19 is shown along with Cu (orange), Zn (gray), Pt (magenta) and Cl (green).

Crystals of cisplatin-treated SOD, suitable for X-ray diffraction analysis, were obtained, by the group of prof. Messori, within two weeks incubation of the protein with a tenfold molar excess of cisplatin; X-ray diffraction data were collected at low temperature and the structure solved through standard methods to 1.8 Å resolution.

Cytochrome c is an intensively studied protein because of its central role in electron transfer in living organisms. Its diverse functional roles and the availability of high-resolution crystallographic data since the early 1970s have contributed to make this protein a paradigm in the study of electron transfer processes. In particular, much interest has focused on the interactions with dfferent kinds of redox partners with which cyt c is known to form stable complexes.

Cyt c is a small electron-carrier heme protein, localised in the mitochondria, that plays a crucial role in the apoptotic pathways. Cyt c is also known to be an excellent ESI MS probe and has been the subject of a number of investigations. This led us to choose cytochrome c as the model protein for our study.

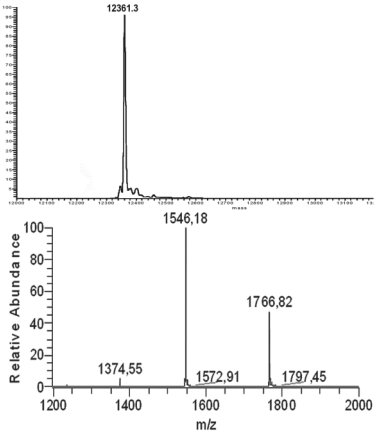

Figure 2.4 ESI/MS deconvoluted spectra (a) and positive-ion mode ESI Mass spectra (b) of cytochrome c.

Cyt c presents, indeed, a number of favourable features: it is a small size protein suitable for ESI MS studies (MW 12362); it shows spectroscopically useful and intense absorption bands in the visible; it possesses a covalently linked heme moiety; it is known to produce well resolved ESI MS spectra in the 1000-2000 m/z region;52,53 (figure 2.4) a few "free" sites are available on its surface that may specifically react with transition metal ions, i.e. two histidines (His 26 and His 33) and one methionine (Met 65).

Ubiquitin has been studied so far exclusively with antineoplastic platinum complexes including cisplatin, transplatin, and asymmetric trans-Pt complexes. Ub is a small protein consisting of 76 amino acids and has a molecular mass of 8565 g/mol (see Figure2.1 for a crystal structure). The sequence of Ub, with the amino acids marked that are the most important nucleophilic sites capable of binding metal centers, is provided in Figure2.1. Ubiquitin fulfills essential functions in eukaryotes; when substrates are marked with a polymer of ubiquitins, they are targeted to a multisubunit ATP-dependent protease while substrates marked with only a few ubiquitins are prone to endocytosis resulting finally in proteolysis. Cisplatin was found to form mono- and bisadducts with Ub of different type, while transplatin forms mainly the monofunctional adduct Ub–[Pt(NH3)2Cl].54,55 The asymmetric Pt(II) complexes were reported to coordinate exclusively to Met1 of ubiquitin, which was proposed to be also the main target for cisplatin, but not for transplatin.

An other protein useful as a model to investigate the interactions of metallodrugs is the hen egg white lysozyme (HEWL). Indeed, lysozyme has a molecular mass of about 14305 Da (129 amino acids), and owing to its small size and to the prevalence of positively charged groups, is a particularly suitable protein for ESI MS investigations as previously shown. Moreover, HEWL is well known among crystallographers as a protein very prone to crystallisation, thus turning out very appropriate for X-ray diffraction studies of its metallodrug adducts. The possible sites available for the interaction with metal complexes are 1 histidine (His 15) and 2 methionine (Met 105, Met 12). The 8 cysteine, instead, are involved in 4 disulfide bonds.

Overall, the studies carried out on metallodrugs and model proteins permit a rather accurate characterisation of their interaction modes at a molecular level. Of course, when carrying out this type of investigations, one must never forget that just very simplified systems were analysed. Indeed, in most cases the various biophysical studies have been mainly conducted on a two component systems containing a single metallodrug and a single protein. Moreover, the investigated protein is in most cases a model protein, usually being of moderate to low molecular weight, water soluble, stable and easy to manipulate.

2.3 Target proteins

Undoubtedly, the study of metallodrug-protein reactions is a very complex investigation field due to the huge number of existing proteins, to their extreme structural diversity and to their supramolecular assembly and compartimentalization within the various subcellular structures. Powerful separation and analytical methods are thus strictly required to unravel the complicate networks of metallodrug-protein interac-

tions; some relevant progresses in this field was eventually achieved following implementation of the latest mass spectrometry techniques and of advanced proteomics and metalloproteomics protocols.

The availability of increasingly sensitive and powerful mass spectrometers is now opening the way to analyze metallodrug adducts with protein of higher size and complexity. Within this frame, development of this work on the interaction of anticancer agent with protein will be the switch from model proteins to true target protein as carbonic anhydrase, thioredoxin reductase (principally involved in the redox metabolism in the cytosol and in the mitochondria) and cathepsins (proteinases that are crucial in certain types of cancer and strictly linked to the processes of invasion and metastasis).

The activity of these families of proteins and their possible inhibition may be directly monitored through spectrophotometric or fluorimetric enzyme activity assays. In particular the studies on the thioredoxin reductase will be performed in collaboration with the group of Dr.Bindoli of the CNR of Padova.

2.3.1 Thioredoxin reductase system

Mammalian thioredoxin reductases (TrxR) are large homodimeric proteins that play a major role in the intracellular redox metabolism, together with a few other systems. Thioredoxin reductases are characterized by a broad substrate specificity and by a well-accessible redox center.68 In mammalian TrxR, this redox center consists of a cysteine-selenocysteine redox pair which approaches the N-terminal active site of the other subunit for electron transfer. Notably, the active site selenolate group, after reduction, manifests a large propensity to react with “soft” metal ions making TrxR a potential pharmacological target for a vast array of metallodrugs. This is most likely the reason why various gold(I) and platinum(II) compounds were earlier reported to be potent inhibitors of mammalian thioredoxin reductase.

Two main forms of TrxR exist in mammalians, namely a cytosolic (TrxR1) and a mitochondrial one (TrxR2). The exact physiological role of these two forms is yet not known. In any case, their main function is the reduction of the 12 kDa disulfide protein thioredoxin (Trx) to the corresponding dithiol species. Apart from being an electron shuttle for ribonucleotide reductase, Trx modulates the activity of a few transcription factors, supports protein biosynthesis and folding, regulates enzyme activities, serves as an anti-oxidant and can act extracellularly as an autocrine growth factor.

The multitude of crucial biological functions performed by the thioredoxin/thioredoxin reductase system render it an attractive “druggable” target. Some recent studies suggested that the development of new TrxR inhibitors might be of interest not only for cancer chemotherapeutics but also for the treatment of a variety of diseases such as rheumatoid arthritis, Sjögren's syndrome, and AIDS.

Furthemore, recent studies suggest that alterations in apoptotic pathways and, in particular, mutations of the p53 gene may result in chemotherapeutic resistance. There is a general agreement on deeming the decreased tendency of the cisplatin-resistant cell to undergo apoptosis76 as a common basis where all the proposed resistance mechanisms converge.

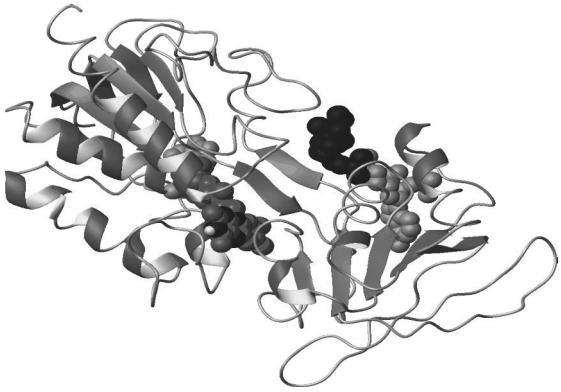

Figure 2.5 Schematic representation of the molecular structure of mammalian TrxR1 visualised with the Swiss-Pdb Viewer software.

Apoptosis is an active death process genetically encoded that can be triggered by a wide variety of extra- and intracellular stimuli. A substantial volume of experimental evidence points to the central role of mitochondria in apoptosis. This includes changes in mitochondrial membrane permeability and electron transport function, and the mitochondrial release of apoptosis-stimulating proteins such as cytochrome c, AIF, and procaspases. Consequently, mitochondria represent an important target for anticancer drugs and, in particular, the mitochondrial thiol-dependent redox systems involving glutathione and thioredoxin can be altered after interaction with several antitumor agents. As a consequence, the inhibition of both cytosolic and mitochondrial thioredoxin reductase can shift the redox balance toward a more oxidized state and hence alter the mitochondrial membrane permeability conditions with the consequent release of the segregated proapoptotic factors. The thioredoxin system, including NADPH, thioredoxin reductase, and thioredoxin, participates in several cell processes including reduction of protein disulfides, removal of hydrogen peroxide through peroxiredoxins, formation of deoxyribonucleotides mediated by ribonucleotide reductase, and regulation of transcription factors.

In particular, thioredoxin reductase is endowed with a flexible C-terminal extension containing a cysteine/selenocysteine redox center that can easily interact with different and chemically unrelated substrates and inhibitors. Among the inhibitors of thioredoxin reductase, gold compounds are very effective and act at nanomolar levels. Auranofin, a coordinated gold(I) compound, is known to react with selenol-containing residues, and to inhibit thioredoxin reductase in near stoichiometric amounts with a formal Ki of 4 nM.86 In addition, gold compounds, successfully used as antirheumatic drugs, were also shown to act as anticancer agents, to inhibit mitochondrial functions, to stimulate the release of cytochrome c, and to induce apoptosis.

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Chapter 3

Outline and aims of the research

3. Research background

The field of investigation concerning metal complexes as potential antitumor agents manifests today a great vitality since the application of very recent investigational methodologies promises substantial progresses in the elucidation of their mechanism of action at a molecular level. The several studies that have appeared during the last 20 years on antitumor platinum metallodrugs revealed the extreme complexity of the molecular mechanisms of metal based drugs that cannot be accounted for only on the basis of a blockade of specific DNA functions. Detailed studies have clearly shown that the formation of platinum-DNA adducts eventually leads to cell death through a complex cascade of biochemical events also involving many specific proteins (e.g. HMG proteins, p53, caspases and others).

On the other hand, a number of direct interactions of platinum compounds with either intracellular or extracellular proteins, have been unambiguously demonstrated.3,4,5 The functional meaning of these interactions is still largely unclear; however, they seem to play key roles in the toxicity and resistance processes as well as in the overall pharmacological mechanism. More in general, for many anticancer metallodrugs (both platinum and non platinum) several interactions with various biomolecular targets, other than DNA, have been unambiguously evidenced, that are of relevance for their global pharmacological and toxicological profile.

Based on these arguments, it is today very important to extend the study of the mechanism of action to the consideration of several biomolecular targets, either intracellular or extracellular, focusing the attention on specific protein targets. In fact, there is today a rather general consensus that it is crucial to acquire specific information on the underlying biochemical processes in order to rationally direct the discovery of novel compounds through a "mechanism oriented" approach.

For this purpose, of particular value are a few X-ray diffraction studies of crystalline metallodrug-protein adducts as well as some ESI mass spectrometry investigations of similar systems in solution. By these methods, accurate information on the nature of the metallic fragments bound to the protein and on their spatial localisation could be obtained.

ESI Mass Spectrometry

Today, thanks to the latest technological improvements, ESI MS represents a very powerful method for the molecular characterisation of metallodrug-protein adducts. A

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series of pioneering studies carried out by Dan Gibson and coworkers during the 1990s and the early 2000s, highlighted the advantages of this method and defined the experimental conditions for its application to simple metallodrug/protein systems. Most of his studies focused on the reactivity of cisplatin and analogues with ubiquitin, taken as the reference model protein. , The careful interpretation of the ESI MS results collected under various experimental conditions allowed Gibson and coworkers to assign the two main platinum binding sites in ubiquitin, to describe the time dependent evolution of the resulting platinum protein adducts and also to monitor their reactivity with other relevant biomolecules that are present intracellualrly e.g. glutathione and various nucleobases.

The high content of structural and functional information that could be derived from those early ESI MS studies prompted us to use a similar approach for the characterisation of our metallodrug-protein systems.

X-ray crystallography

Although single crystal X-ray diffraction still represents the election tool to obtain high quality structural information on proteins, especially for those of medium to large size, until now very few crystal structures have been solved to a high resolution for metallodrug-protein adducts. This situation may be principally ascribed to the intrinsic difficulty in obtaining good quality crystals for metallodrug/protein adducts.

Some additional structures, somehow connected to our topics, had been obtained by Jaouen and coworkers working on lysozyme modification by specific organometallic species. In contrast, a quite conspicuous number of crystallographic structures were available in the HAD data bank in relation to the heavy atom replacement methodology. However, these latter structures deal only marginally with effective metallodrugs; moreover, in several cases, details on the coordination environment of the bound metal are not available.

Table 2.1 Crystal structures of pharmacologically relevant metal complexes bound to proteins.

Metal complex/protein adduct	Metal	Resolution (Å)	Metal binding-site	PDB	year of publication
cisplatin/beSOD	Pt(II)	1.8	His 19	2AEO	2006
cisplatin/HEWL	Pt(II)	1.9	His 15	2I67	2007
NAMI A/human lactoferrin	Ru(III)	2.6	His253	-	1996
KP1019/human lactoferrin	Ru(III)	2.2	His253	-	1996
HInd[RuIndCl$_4$]/human lactoferrin	Ru(III)	2.4	His253	-	1996
[(η^6-p-cymene)RuCl$_2$H$_2$O/HEWL	Ru(II)	1.6	His 15	1T3P	2004
Au(PEt$_3$)Cl/cyclophilin-3	Au(I)	1.85	His133	1E3B	2000
Gold thiomalate/human cathepsin K	Au(I)	2.0	Cys 25	2ATO	2007

[a] Ind = indazole. [b] PEt$_3$ triethylphosphine.

Thus, if one discards crystal structures of the HAD data bank and those related to the work of Jaouen, only 8 crystal structures are left for adducts of proteins with platinum, ruthenium or gold metallodrugs (Table 2.1). Notably pdb coordinates have been deposited only for 5 of these 8 adducts. It turned out that no crystal structure was available for any adduct of cisplatin with proteins.

3.1 Aim of the Project

Within this frame, the aim of this research project was the characterisation of binary adducts formed between various kinds of metal compounds and a number of biomacromolecular targets, in order to obtain specific information concerning the molecular mechanisms of action and the structure/function relationships of these metallodrugs. These studies have taken advantage of the above mentioned techniques.

In particular, we have studied some model proteins and a few proteins that are believed to represent effective pharmacological targets for cytotoxic metal complexes, as thioredoxin reductase. Such information will turn out to be crucial for the design and the obtainment of new compounds with improved pharmacological profiles.

In detail, the project was organised in the following phases:

Phase 1. Structural and functional characterization of platinum(II)-protein adducts

In a first step, to unravel the interactions of metal-based anticancer drugs with proteins, we selected the ESI MS techniques and optimized this method, applying it to selected model proteins. Then we made reacting these model systems with the classical anticancer drug cisplatin and with its derivatives (carboplatin, transplatin, oxaliplatin). In detail, first we have characterised the cytochrome c/platinum system and than the lysozime/platinum adducts, both with ESI MS analysis.

Upon careful optimization of the experimental conditions, very satisfactory S/N ratios were obtained for all shown ESI MS profiles; accordingly, deconvoluted spectra of high quality were obtained that revealed formation of a variety of platinum-protein adducts. The stoichiometry of the adducts and the nature of the protein-bound metal fragments were unambiguously determined. Different kinds of adducts were observed depending on the selected complex, proving the interaction of the various platinum compounds with the selected model protein (cytochrome c and lysozime). Remarkably, the reactions with platinum drugs produced, in all cases, the appearance of new ESI MS features that are diagnostic of the formation of stable platinum-protein adducts. However, binding of platinum to the protein does not bring about any substantial modification of the distribution pattern of the multicharged ions implying that the overall protein conformation is scarcely affected.

Under the applied experimental conditions, the four selected compounds turned out to exhibit a roughly similar pattern of reactivity with horse heart cytochrome c (cyt c). This finding was soon of particular interest and novelty, being in striking contrast with current opinions concerning the comparative reactivity of the investigated platinum drugs. Indeed, the four platinum compounds that were selected for our study are commonly known to exhibit greatly different stability and reactivity patterns under physiological-like conditions. For instance, carboplatin and oxaliplatin have been re-

ported to hydrolyse about 100-fold less rapidly than cisplatin. The far higher stabilities of carboplatin and oxaliplatin are reflected in a lower reactivity with DNA and with other proteins. Thus, it was quite surprising finding that all platinum compounds tested in our study produced substantially similar levels of cyt c platination. Accordingly, we deduced that cyt c should play a major role in enhancing the reactivity of the kinetically stable carboplatin and oxaliplatin compared to cisplatin and transplatin.

In a latest work we have study in depth this improve reactivity of carboplatin with cyt c, that produce stable platinum/protein complexes. ESI MS studies allowed us to characterise, at the molecular level, the two major carboplatin/cyt c complexes resulting from the above reaction. We have also evaluated the ability of these adducts to react with 5'GMP giving rise to the respective cytochrome c/carboplatin/5'GMP ternary complexes. Additionally, in order to identify the platinum(II) binding sites on cyt c, we analysed the primary sequence of the protein and its crystal structure and than ESI MS measurements recorded on enzymatically cleaved samples of platinated cytochrome c adducts were performed. This analysis had supported the view that Met 65 might represent the primary binding site for platinum drugs on horse heart cyt c. The mechanistic implications of the obtained findings are discussed.

Afterward, we applied the same investigative approach to platinum adducts of hen egg white lysozyme (HEWL). Even in this case ESI MS measurements turned out very valuable to monitor the process of metallodrug-lysozyme adduct formation and to elucidate the exact nature of the protein bound metallic fragments. We have learned that, under the employed solution conditions, platinum-HEWL adduct formation is rather slow, that cisplatin is by far the most efficient platinum compound in producing HEWL platination, that monoplatinated species are the predominant ones, again suggesting the presence of a highly preferential platinum binding site. Based on the crystal structure of cisplatin HEWL, reported above, this primary binding site should be straightforwardly assigned as the imidazole ring of His 15.

Then, to have a better description of the interactions at a molecular level, we applied X-ray crystallography, solving the structure of the cisplatin/lysozyme adduct. In this structure solved to 1.9 Å resolution, the platinum atom is apparently bound to the the Nε of His 15 and, to the nitrogens of two ammonia molecules in cis. The fourth ligand is not detectable: it might well correspond to a loosely bound/disordered platinum coordinated water molecule. No other significant modifications of the electron density map of the protein surface were observed ruling out the presence of additional (secondary) binding sites; for instance the two methionine residues (Met 12 and Met 105) that commonly represent preferred anchoring sites for platinum(II) compounds turned out to be unmodified.

It is of interest to observe that protein platination takes place predominantly to a single protein site; occupancy of secondary site is not detected at all within the resolution limits of the technique. Also the Nε of histidines serves as primary anchoring sites for platinum(II). Unfortunately several other attempts to obtain crystals suitable for X-ray diffraction were unsuccessful for other platinum derivatives highlighting the intrinsic risks of this approach.

Very recently, we have extended the ESI MS approach to monitor the reactions of some novel anticancer platinum(II) iminoether complexes, namely trans- and cis-EE (trans- and cis-[$PtCl_2${(E)-HN=C(OCH_3)CH_3}$_2$], respectively) and trans- and cis-Z (trans- and cis-[$PtCl_2(NH_3)$}{(Z)-HN=C(OCH_3)CH_3}], respectively), with cyt c. Our investigation was independently supported by NMR, ICP OES, and absorption electronic spectroscopies.

From this study it has emerged very clearly that interactions with this cyt c do profoundly alter the intrinsic reactivity of the various platinum iminoethers, leading to the observation of rather unexpected chemical transformations at the level of the platinum ligands. In addition, the kinetics of degradation of the platinum complexes could be measured and found to be largely influenced by the interactions with this protein. Remarkably, a profoundly different pattern of reactivity was identified for the trans isomers with respect to the cis. Valuable information for the platinum binding site assignment was achieved through a partial proteolysis experiment by using the endoproteinase Asp-N. The comparative analysis of the obtained results with those previously reported for classical platinum(II) anticancer drugs made us confident that Met 65 is the major binding site for platinum(II) iminoethers on cyt c.

Phase 2. In vitro interactions of ruthenium complexes and model proteins: an ESI MS study

As the ESI MS method provided so informative results in the case of platinum-protein adducts we thought that it might be pair wise effective for the characterisation of adducts formed between proteins and some important non platinum metallodrugs.

Within this frame we started a study on a specific class of Ru(II)-arene complexes (RAPTA compounds). All these compounds share a common structural motif consisting of a ruthenium(II) center bound to both an arene (cymene in this case) and to a 1,3,5-triaza-7-phosphaadamantane (PTA) ligand. They only differ in the nature of the ligands located at the two remaining coordination positions. The first representative member of this family is [Ru(η^6-cymene)(pta)Cl_2] (RAPTA-C). Notably, replacement of the two chloride groups with bidentate ligands [either oxalate -to form Ru(η6-cymene)(pta)(C_2O_4) (oxalo-RAPTA)- or cyclobutane dicarboxylate -to give Ru(η^6-cymene)(pta)($C_6H_6O_4$) (carbo-RAPTA)]- greatly reduces the rate of aquation, thus modifying their overall solution behaviour, without affecting cytotoxicity. The three investigated complexes essentially manifested a similar cell-growth inhibition activity against a number of representative cancer cell lines. The binding of a wide range of RAPTA derivatives to oligonucleotides was formerly studied but no direct correlation between oligonucleotide binding and cytotoxicity could be established. This finding might suggest that protein targets are of great importance in producing the observed cytotoxic effects.

In our study the three "RAPTA" complexes were challenged with either cyt c or HEWL and the resulting reaction products analysed by ESI MS. Remarkably, the obtained results could be subsequently confirmed by high resolution mass spectrometry

measurements, carried out on an LTQ Orbitrap instrument (Thermo, San Jose, CA) equipped with a conventional ESI source.

More recently we have extended our ESI MS approach to the well known antimetastatic Ruthenium(III) complex NAMI A developed by Mestroni, Alessio and Sava in Trieste. Some previous spectroscopic work had been directed to the analysis of the interactions of NAMI A with typical serum proteins like serum albumin and serum transferring. Although the main features of NAMI A/serum protein interactions were determined in those initial studies, molecular details of the binding processes could not be fully elucidated due to the relatively high molecular weight of the mentioned proteins and to failure to obtain high resolution X-ray crystal structures for the resulting ruthenium-protein adducts.

Thus, we reacted NAMI A either with cyt c or HEWL and monitored the analysed the resulting products through ESI MS. The masses of the various protein-bound ruthenium containing fragments could be determined, in most cases, with high resolution, and the fragments assigned to specific molecular structures.

It is worth mentioning that in this work we also monitored the reactivity of NAMI A with the above proteins through a variety of independent physico-chemical methods including optical spectroscopy, 1H NMR and ICP OES. The combined use of the mentioned analytical techniques complemented and confirmed the information obtained through ESI MS.

Quite unexpectedly, two substantially different modes of metallodrug-protein interaction clearly emerged for NAMI A in the two cases. In fact, lysozyme gave rise, predominantly, to non-covalent binding with either intact or mono-hydrolyzed NAMI A, most likely mediated by electrostatic interactions. Protein binding appeared to be largely reversible. Remarkably, these interactions greatly slowed down intrinsic NAMI A degradation processes.

In contrast, cyt c was found to enhance NAMI A degradation by facilitating the progressive detachment of the various ligands from the ruthenium center. Most likely, this process is favoured by an initial electrostatic interaction between the negatively charged NAMI A "core" and this small basic protein (indeed, similarly to lysozyme, cyt c is a highly cationic protein at physiological pH with a pI of ~9.59 which is very prone to interact with anions). Such initial interaction is then progressively replaced by coordinative binding of the ruthenium(III) center to the protein. Eventually, a highly degraded ruthenium containing species, in which most of the original metal ligands have been lost, was found to remain attached to the protein.

Finally, a novel "Keppler-type" ruthenium(III) compound, namely trans-[bis (2-amino 5-methylthiazole) tetrachlororuthenate(III)] (PMRU27), of potential interest as an anticancer agent, was structurally characterised and its solution behaviour analysed. Its interactions with various representative proteins were studied, among which the selenoenzyme thioredoxin reductase, an emerging target for anticancer metallodrugs. Remarkably, a selective inhibition of the cytosolic form of the enzyme was observed, this being the first report of thioredoxin reductase inhibition by a ruthenium compound.

Phase 3. Gold compounds as pharmacological agents: relevance of gold-protein interactions for their mechanism of action

The renaissance of interest for gold compounds as potential anticancer metallodrugs has resulted, in the course of the last decade, in the synthesis of a number of structurally diverse gold(I) and gold(III) species, endowed with sufficient chemical stability and with relevant antiproliferative activities. Most of the mechanistic studies carried out on cytotoxic gold compounds were generally referred and compared to the behaviour of cisplatin, for which DNA is thought to be the major target.

However, it has emerged quite clearly from the experimental results collected so far that the respective molecular mechanisms are rather distinct. Overall these mechanistic studies suggest that alternative biochemical processes must be operative, most likely associated to selective modification of some crucial proteins. In this respect it is worth noting that gold(I) and gold(III) compounds are known to target, rather strongly and selectively, thiol and imidazole groups of proteins (as well as selenol groups).

Then, to further elucidate these issues and describe the main cellular effects induced by novel organogold(III) compounds, new experiments were carried out during this work. Specifically, the following organogold(III) compounds were selected for the present investigation: [Au(bipydmb-H)(OH)][PF_6] (AubipyC), Au(bipydmb-H)(2,6-xylidine-H)][PF_6] (AuXyl) (in which bipydmb = 6-(1,1-dimethylbenzyl)-2,2'-bipyridine), and [Au(pydmb-H)($AcO)_2$] (3) (in which pydmb = 2-(1,1-dimethylbenzyl)-pyridine). These compounds were previously characterized and found to cause significant antiproliferative effects on the human tumor cell lines A2780, MCF7, HT29, and A549.

We analyze the effects produced by these organogold(III) compounds on ovarian A2780 carcinoma cells, either sensitive (A2780/S) or resistant (A2780/R) to cisplatin, in terms of cytotoxicity, cell cycle modifications, and induction of apoptosis. The effects were investigated in comparison to cisplatin and oxaliplatin. The tested compounds produced significant antiproliferative effects and promoted apoptosis to a greater extent than platinum drugs while causing only modest cell cycle modifications. The mechanistic implications of these findings are discussed: mitochondrial pathways are proposed to be directly involved in the apoptotic process in relation to selective inhibition of thioredoxin reductase.

In this frame, a series of six dinuclear gold(III) oxo complexes with bipyridyl ligands, of general formula [Au_2(N^N)$_2$(μ-O)$_2$][PF_6]$_2$ [where N^N = 2,2'-bipyridine (Auoxo1), 4,4'-di-tertbutyl- (Auoxo2), 6-methyl- (Auoxo3), 6-neopentyl- (Auoxo4), 6-(2,6-dimethylphenyl)- (Auoxo5), 6,6'-dimethyl-2,2'-bipyridine (Auoxo6)] were prepared and characterised, and their antiproliferative properties evaluated in vitro toward the reference A2780 human ovarian carcinoma cell line.

The crystal structures of four members of this series of gold complexes, namely Auoxo1, Auoxo3, Auoxo5 and Auoxo6 were solved and the respective structural parameters comparatively analyzed.

The chemical behaviour of these compounds in solution has been studied in detail, in particular focusing on the electrochemical properties. Some initial correlations are

proposed among the structural parameters, the chemical behaviour in solution and the cytotoxic effects.

Notably, we found that, while five compounds manifested moderate cytotoxic properties (with IC50 ~ 10-30 μM), the sixth one (Auoxo6), which shows the largest structural deviations respect to the model compound Auoxo1, has also the highest oxidizing power, the least thermal stability and the greatest cytotoxic activity (~ 5-15 times more active against both cell lines) and will merit further pharmacological studies, in fact the positive correlation existing between the oxidizing power and the antiproliferative effects seems to be of particular interest. Moreover, the electronic structures of these compounds were extensively analysed by means of Hybrid-DFT methods, and the effects of the various substituents on reactivity predicted; overall, a very good agreement between theoretical expectations and experimental data has been achieved. On turn, theoretical predictions offer interesting hints for the design of new and more active binuclear gold(III) compounds.

Finally, the interactions of two more representative members of this series (Auoxo1 and Auoxo6) with a few model proteins (serum albumin, cytochrome c, ubiquitin) and with calf thymus DNA were analysed in detail by various spectroscopic methods and by ESI MS. Both tested compounds manifested a high and peculiar reactivity toward the mentioned model proteins; specific differences were detected in their reactivity with DNA.

In particular, ESI MS spectra were recorded after reacting cyt c with the various Auoxo complexes, working at 1:1 Auoxo/cyt c ratios. After 12 hours incubation, cyt c was extensively ultrafiltered against the ammonium carbonate buffer and the ESI MS spectra of the upper fractions recorded. In all cases, the final deconvoluted ESI MS spectra provided clear evidence of adduct formation. Remarkably, a number of peaks were observed corresponding to formal binding to the protein of a number of Au+ ions (ranging from 1 to 4). A similar behaviour had previously been reported by Sadler and coworkers in the case of the adducts of gold(I) triethylphosphine chloride with cyclophilin.

It is remarkable that no sign of the bipiridyl ligand coordinated to gold was found anymore implying that the reduction process causes complete disruption of the starting dinuclear compound with cleavage of the oxo-bridges, release of the bipyridyl ligand and protein binding of the isolated gold ions.

The mechanistic implications of these results are discussed.

Similar studies were also started on the interactions of the gold(I) compound auranofin with another model protein, the zinc-enzyme carbonic anhydrase and with the target enzyme thioredoxin reductase.

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Chapter 4

Results

4. Structural and functional characterization of platinum(II)-protein adducts

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4.1 In vitro interactions of ruthenium complexes and model proteins: an ESI MS study

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Chapter 5

Conclusions

The first two phases of this work were focused on the molecular characterisation of adducts formed between a number of platinum and ruthenium metallodrugs and a few proteins, either models or targets. Valuable structural and functional information on these adducts was derived from X-ray diffraction and ESI MS techniques.

The application of X-ray diffraction and ESI MS techniques for the characterisation of metallodrug-protein adducts has undoubtedly allowed a significant progress in this specific research area. Indeed, thanks to the results provided by these two potent physicochemical methods, the molecular mechanisms of the reactions of metallodrugs with protein targets could be described at least in selected cases with a high accuracy.

In any case, on the ground of the obtained results, a rather satisfactory and comprehensive description of the reactivity of metallodrugs with proteins can be inferred that might be of broader validity. The main features of such reactivity are outlined below.

Metallodrugs behave very frequently as pro-drugs. This means that they must necessarily undergo an activation step, in most cases a simple aquation reaction, before they can react with protein targets. Generally, this activation step represents the rate limiting step; however we have also shown that the kinetics of this activation step may be greatly influenced by a direct interaction of the metallodrug with proteins. This is the reason for the unexpectedly similar reactivity profiles of carboplatin and cisplatin with cytochrome c, earlier mentioned.

The resulting "activated metallodrugs" usually contain a weakly coordinated water molecule that is easily removed and replaced by a stronger ligand provided by the protein itself. Only a few protein residues perform this function with the "soft" metal ion here considered, mainly histidines, methionines, cysteines through nitrogen or sulphur donors. This second ligand substitution reaction leads to the formation of the so called metallodrug/protein complex or adduct, the main object of our investigations. Notably, both X-ray diffraction and ESI MS studies converge in showing that, in spite of the large number of potential donors on the protein surface, adduct formation takes place preferentially only in a few positions, implying a rather high selectivity in metal binding.

The functional characterisation of the newly formed entities -i.e. the metallodrug protein adducts- is of extreme importance in relation to their possible biological roles. Indeed, if the adduct is shown to be devoid of any further reactivity we can assess quite safely that the reaction has led to inactivation of the metallodrug (provided that the protein itself is not an important biological target). Conversely, if the adduct conserves

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the capacity of further reacting with other biomolecules and/or of transferring the metallic fragment to other species, one can state that the formed adduct is still a biologically active species and that it may also serve as a reservoir of the metallodrug itself. The ESI MS method is very appropriate for this specific purpose. For example, we have shown, in our study, a residual important reactivity of the ruthenium(III) compound NAMI A after binding to cytchrome c.

In the phase 3 of this work, we have synthesized new gold(III) compounds as cytotoxic agents. In detail, wee have reported that these structural diverse gold(III) complexes exert outstanding antiproliferative effects when tested in vitro against various tumour cell lines. The observed biological effects are most likely the consequence of innovative and distinct molecular mechanisms in dependence of the peculiar coordination and redox chemistry of the gold(III) center and of the nature of its ligands. DNA seems not to be a primary target for most novel cytotoxic gold(III) compounds. In contrast, the important reactivity detected toward model proteins favours the idea that the cytotoxic effects of gold(III) complexes are primarily mediated by interactions with proteins targets. For instance, relevant anti-mitochondrial effects, probably arising from direct inhibition of thioredoxin reductase were highlighted, that activate the apoptotic cascade after triggering mitochondrial cyt c release.

We have also shown here that the reactivity of gold(III) compounds with proteins may be accurately monitored, at the molecular level, through physicochemical studies of appropriate model systems. In this respect, ESI MS has again turned out to be a very powerful method to follow formation of protein adducts and identify the nature of protein bound metal fragments. In the reported examples, concomitant redox and metal binding processes seem to be crucial in producing specific protein damage as documented by the case of cytochrome c. Overall, in view of their peculiar chemical and biological properties, it seems that these novel cytotoxic gold(III) complexes have the potential of being further developed and exploited as experimental anticancer drugs

In conclusion, the studies on metallodrugs and model proteins permit a rather accurate characterisation of their interaction modes at a molecular level.

The two high resolution techniques used here are independent but highly complementary in that X-ray crystallography provides a full description of the structure of the metallodrug-protein adducts, while ESI MS offers valuable information on the temporal evolution of these adducts in physiological-like conditions. Putting together these two pieces information usually results in an exhaustive structural and functional picture of the analysed systems and of the underneath reactions. Notably, one major limitation usually arises from the intrinsic difficulty in obtaining good quality crystals for X-ray diffraction analysis. Indeed, crystal structures of metallodrug-protein have been solved until now only in a very limited number of cases. In these cases, ESI MS may be complemented by independent information achieved by application of other physicochemical techniques such as NMR. Another limitation is represented by the fact that the two mentioned techniques have rather different solution requirements that might affect in some cases the resulting reactivity.

Finally, when carrying out this type of investigations, one must never forget that just very simplified systems are analysed. Indeed, in most cases the various biophysical

studies have been mainly conducted on simple two component systems containing a single metallodrug and a single protein. Moreover, the investigated protein is in most cases a model protein, usually being of moderate to low molecular weight, water soluble, stable and easy to manipulate.

Real cellular systems are of course extremely more complicated as they may contain thousands of different proteins at highly variable concentration and a conspicuous number of low molecular weight components, most of them in relatively high concentration. In addition, these various components are highly organised and compartmentalised within the various intracellular structures thus offering a peculiar spatial distribution inside cells. It is obvious that studying the interaction of metallodrugs with proteins inside cells represents today a formidable task for researchers. Nonetheless, we believe that the reactivity patterns that we have identified and characterised for the simplified systems may be a good reference in the study of complexes cellular systems. In our opinion it is mandatory to extend as soon a possible the biophysical studies we have outlined above to system of higher complexity in order to ascertain the extent to which the reactivity models defined in purified systems conserve their validity within the real cellular world. A possible compromise between these two extremes might be that of investigating the behaviour of metallodrugs within cell homogenates; the new and potent analytical methods offered by the so called "omic sciences", for instance the methods of metallomics, may be of great advantage for these further advancements.

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