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Interactions of antitumoral platinum-group metallodrugs with albumin

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Contents

Abstract

A full understanding of the modes of action of the metal-based antitumoral drugs requires the study of their interactions with all possible biological targets, including aminoacids, hormones, peptides and proteins. Albumin is the most abundant plasma protein and it is reasonable to expect that any injected metal drug will present some kind of interaction with this macromolecule, which could crucially determine its bioavailability and toxicology. However, relatively few detailed mechanistic studies have been performed on such interactions, in comparison to studies with DNA and nucleobases. In this work is presented a review of the research on reactions of platinum(II) and (IV), gold(I) and (III), ruthenium(III) and rhodium(II) antitumoral compounds with serum albumin. Generally, platinum and gold compounds are found to react with S-donors such as methionine and the Cys34 residues of albumin, the latter being the most abundant free thiol in blood plasma. Complexes of ruthenium and rhodium are thought to react mainly through coordination with imidazole groups from histidine residues.

Keywords: Albumin; Antitumor; Platinum; Gold; Ruthenium; Rhodium

1. Introduction

The study of the medical applications of metal complexes is one of the most integrative investigation areas in modern inorganic chemistry, leading the researcher to combine data from the rich fields of metal chemistry and the control and maintenance of life processes [1–5].

Since it was suggested that cisplatin, one of the world's most important chemotherapeutics, acts mainly

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; Cys, cysteine; His, histidine; HSA, human serum albumin; kDa, kilodalton; Met, methionine; rHA, recombinant human albumin; Trp, tryptophan.

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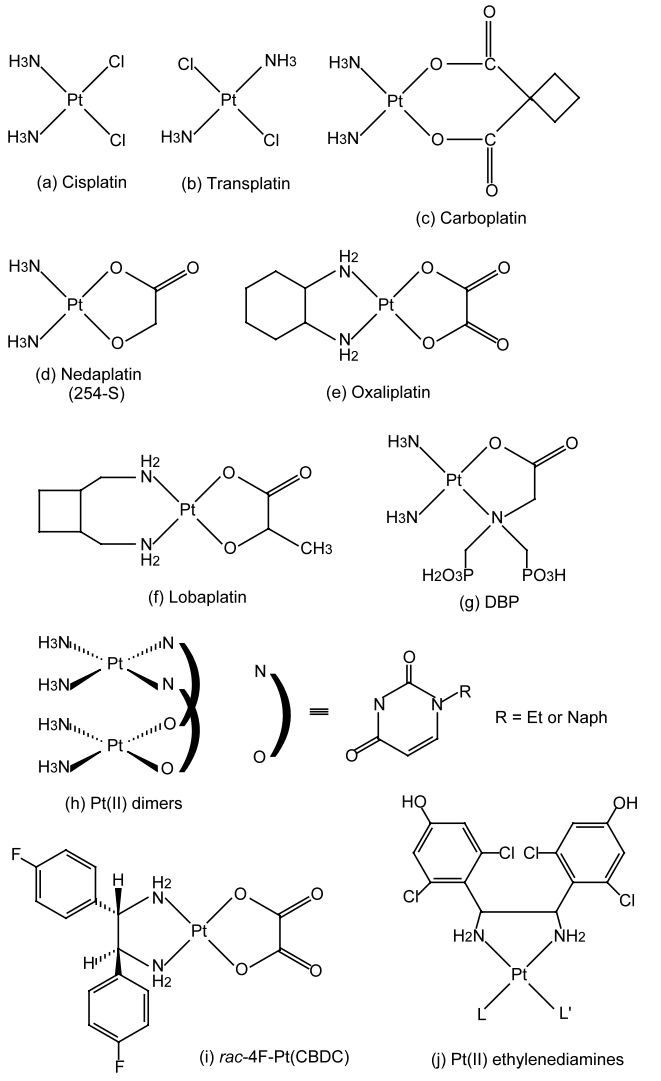


Fig. 1. Structure of some antitumoral Pt(II) complexes.

through induction of DNA unwinding and DNA-protein binding [6], the focus of a great part of the research has been to seek other metal complexes (Fig. 1) with similar but more selective action. There is, however, substantial biomedical evidence as well as chemical reasons for the involvement of non-DNA targets that could determine the antitumor activity of platinum-group metallodrugs, including cisplatin. From a purely chemical point of view, these metals acting as 'soft' Lewis acids should be expected to form stable complexes with S or N donors in peptides and proteins. Thioether sulfur reacts more rapidly with aquo-platinum species than thiol groups (direct coordination is faster than ligand exchange), although Pt–S covalent bonds tend to be much more stable and irreversible. This binding eventually would lead to altered protein conformation and changes in biological activity, especially when enzymatic reactions are affected (for a review see [7]). Among the possible non-DNA targets, is albumin.

Albumin is the most abundant plasma protein, amounting ca. 52% of its proteic composition. In normal individuals, albumin is present in concentrations of 40 mg ml^{-1} ($\sim$0.6 mM; $M_r = 66$ kDa) [8]. Its physiological functions include the control of osmotic blood pressure, the transport, metabolism and distribution of several endogenous or exogenous substances such as hormones, aminoacids, fatty acids, metal cations and drugs, deactivation of free radicals in the extracellular medium, and providing a source of aminoacids for protein synthesis after hydrolysis [9–11]. Albumin offers the important possibility of dissolution in the biological medium for highly hydrophobic substances [12,13].

Human serum albumin (HSA) consists of a single chain of 585 aminoacids organized in three similar domains (I, II and III), each of which contains two subdomains (IA, IB, etc). Its tridimensional structure has been extensively studied [10,14]. At physiological pH, albumin presents two structural isomers, N and B. It is basically a helical protein (α-helix content of 67%), the helices being bound by 17 disulfide bridges and leaving only one free thiol (Cys34) in a crevice, which has a strong affinity for 'soft' acids like Au(I), Ag(I), Hg(II), Cd(II) and, to a lesser extent, Cu(II). Other metal ions such as Cu(II) and Ni(II) bind to other specific sites throughout the protein, especially His3 [15,16]. A plethora of ligands can reversibly bind to albumin at very fast reaction rates, not influenced by enzymes [17]. Albumin contains only one tryptophan (Trp) residue, in position 214. Mammal albumins are highly conservative; HSA shares 75% identity with its bovine or equine analogue [10,11].

The interactions of cisplatin with serum albumins have received the attention of the scientific community studying antitumoral metallopharmaceutical pharmacokinetics and structure-activity relationships. Notwithstanding, the precise mechanisms of these reactions are only recently being clarified. Pt(IV) bioactive complexes are generally believed to act as prodrugs of Pt(II) complexes, after fast reduction in vivo, and albumin sulfhydryl could play a major role in this activation (see for example [18,19]). Gold complexes are often employed in the treatment of rheumatoid arthritis, but several of them in which gold is in the oxidation state + 1 or +3 have been found to exhibit some cytotoxic and in vivo antitumor activity (for reviews, see [20,21]). More than 80% of the circulating gold in patients is albumin-associated [22] and the interactions of only a few of these complexes have been studied exhaustively, namely the phosphine-derivatives of Au(I). Active ruthenium complexes have also been pursued, but there is doubt regarding the role of DNA binding for cytotoxicity. Other metabolic pathways and biological targets were suggested to account for this activity of ruthenium complexes [8,23–25]. The same could be said of the rhodium(II) carboxylates which, despite numer-

ous studies on their enzyme-inhibiting and DNA-binding properties [26], are subject to extensive ligand binding in the bloodstream. To none of these complexes it is determined whether or not the interaction with albumin has any effect on their antitumoral activities.

In this review the interactions of antitumoral metallodrugs (based on platinum, gold, ruthenium and rhodium) with albumin will be further detailed.

2. Platinum compounds

2.1. Pt(II) complexes

A comprehensive study of the binding of cisplatin and ^{15}N-cisplatin to both normal and recombinant HSA (rHA) have been performed by means of ^{1}H,^{15}N-NMR, gel chromatography and selective aminoacid blocking [27]. Recombinant albumin is similar to serum albumin but has a larger thiol content ($\sim$0.9 mol SH per mol^{-1} rHA and ca. 0.5 mol SH per mol^{-1} HSA) and is structurally more homogeneous. A schematic view of the stepwise reactions is shown in Fig. 2.

An interesting finding was that the blocking of Met residues by iodomethane, a modificator of Met residues, hampered the reaction of cisplatin with the protein to a larger extent than blocking or modifying His or Cys34 residues, the latter being commonly assumed to be the main platination site of HSA [28]. Spectroscopic and

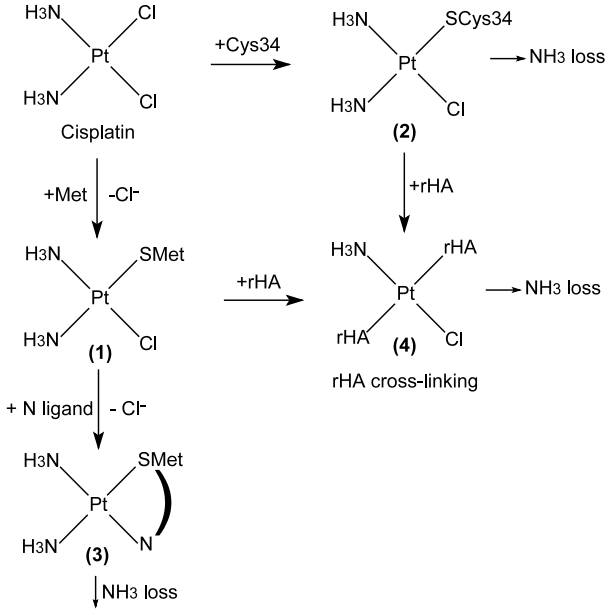


Fig. 2. Stepwise interaction of cisplatin with rHA. Cisplatin initially binds to S-donor residues of the protein (**1** and **2**). The major adduct is a single bifunctional complex of the formula $[Pt(NH_3)_2]^{2+}$ involving S and N donor groups (e.g. **3**). The second binding site involves a S-donor and contains a rather nonreactive Cl^- (**1**). The third type of site is similar but could undergo fast loss of NH_3 (**2**). Cross-linking leading to *trans* compounds (**4**) is also observed (adapted from [27]).

chromatographic data suggested the formation of a S,N-macrochelate, probably involving the surface-exposed Met298 residue, along with monofunctional adducts with other Met residues and Cys34. Met298 was speculated to be the main cisplatin-binding site of HSA [27]. It is clear that different ways to prepare the cisplatin–HSA adduct would lead to different binding mechanisms, which could account for the inconsistencies regarding the clinical activity of these adducts (see below).

Neault and Tajmir-Riahi reported FTIR experiments of the interaction of cisplatin with defatted HSA [29]. These authors monitored the protein Amide I (1656 cm^{-1}, mainly C=O stretch), Amide II (1542 cm^{-1}, C–N stretch coupled with N–H bend) and C–S stretch and C–S–H bending modes below 1000 cm^{-1}, obtaining evidence for direct coordination of the Pt cation to the polypeptide C–N, C=O and sulfur donor groups, which could be additional platinum binding sites in the protein. Infrared measurements have proved to be useful also in determining the changes in the protein secondary structure (Table 1). Even at low cisplatin:HSA ratios the alterations are pronounced, and attributable to the coordination of Pt to C–N, C=O or sulfur donors. An overall binding constant was determined spectrophotometrically for the reaction

$$\mathrm{HAS} + \mathrm{cisplatin} \rightleftharpoons \mathrm{HAS : cisplatin}$$

as being $K = 852$, which is somewhat weaker than other albumin-drug adduct formations [29].

Ohta and colleagues have studied the effects of cis- and transplatin on the conformation of defatted HSA (Table 1) [30,31]. Transplatin is generally assumed to be non-active, but there is evidence showing that this isomer can exhibit some antitumoral activity [32]. The number of bound complexes per mol of HSA after 6 days incubation with 20–40-fold excess of the platinum complexes over HSA was larger for transplatin than for cisplatin, in accordance with previous reports [28]. Since Cys34 is not involved in transplatin binding to HSA ([28] and references therein), this could indicate the presence of other platination sites such as Met, His or S–S bonds. The role of disulfide bridges in the stabilization of HSA structure after cis- and transplatin reaction was, therefore, studied by a number of methods [30,31]. Under the same incubation conditions above, it was found that cisplatin and transplatin cleave 1.2–1.6 and 2.1–3.4 mols of S–S bonds per mol of HSA, respectively. These results showed that the *trans* isomer is more reactive than the *cis* complex and markedly interfered with protein secondary structure (Table 1). Similar results have been obtained upon reaction with cisplatin, even though earlier reports on the interaction of equimolar amounts of cisplatin and HSA accounted for no structural alterations or decreases in tryptophan or warfarin binding [33]. Since the 17 S–S bonds of HSA

Table 1
Structural damage (as indicated by loss of helicity) and fluorescence quenching in albumin provoked by some antitumor metal complexes

Complex	Molecules bound	α-helix decrease (%) (complex:protein)	% of initial fluorescence (complex:protein)	Obs.	Reference
Cisplatin	–	2 (3×10^{-4})	–	defatted HSA	[29]
	–	10 (3×10^{-1})	–	defatted HSA	[29]
	5–10[a]	18 (60)	–	defatted HSA	[30]
	4–6.3[b]	15[c] (20)	55[d] (20)	HSA	[36]
Transplatin	5–10.5[b]	40[c] (20)	45[d] (20)	HSA	[36]
	12–21[a]	20 (40)	32 (40)	defatted HSA	[31]
DBP	1–4[b]	~0[c] (20)	85[d] (20)	HSA	[36]
Him-*trans*[$RuCl_4Im_2$]	5	15 (1)	20–25 (5)	HSA	[100]
Hind-*trans*[$RuCl_4(Ind)_2$]	4	10 (5)	10 (4)	HSA	[102]
$Rh_2(CH_3CO_2)_4$	8	10 (10)	–	defatted HSA	[107]
	8	~0 (15)[e]	45 (15)[f]	HSA	[111]
$Rh_2(CH_3CH_2CO_2)_4$	8	1 (16)[e]	36 (16)[f]	HSA	[111]
$Rh_2(CH_3CH_2CH_2CO_2)_4$	8	7 (11)[e]	30 (11)[f]	HSA	[111]
$Rh_2(CF_3CO_2)_4$	8	10 (16)[e]	20 (16)[f]	HSA	[111]
$Rh_2(CF_3CONH)_4$	8	~0 (15)[e]	27 (15)[f]	HSA	[111]
$[Rh_2(CH_3CO_2)_2(bpy)(H_2O)_2](CH_3CO_2)_2$	6–7	15 (10)	20 (1)	HSA	[109]

[a] After 6 days, molar ratios from 20:1 to 40:1 (complex:albumin).
[b] 1 and 14 days incubation.
[c] After 14 days.
[d] After 7 days.
[e] 24 h, 37 °C.
[f] Unpublished data.

are crucial for protein structure stability, it was assumed that the loss of helical content was a function of the number of cleaved S–S bonds. Differently from cisplatin, transplatin fluorescence quenching leads to a blue shift from 350 to 346 nm in the emission spectra, which was explained in terms of the formation of a molten globule-like state of HSA in which hydrophobic clusters can form upon denaturation. Cisplatin interaction induced a red shift through unfolded states. A further confirmation of the important alterations in protein conformation induced by the reaction with excess transplatin was given by the loss of warfarin binding ability to HSA (warfarin is a high affinity probe for the region of Trp214 of HSA) [30,31]. Although sometimes pronounced, these alterations would presumably account for no important clinical problems since serum albumin is in a large excess over the metallodrugs.

The reaction of cisplatin with S–S bridges in HSA has also been studied by means of adsorptive voltammetry [34]. At [HSA] ca. 10^{-7} M in phosphate buffer (pH 7.4), albumin presents two current peaks at −0.44 (A′) and −0.56 V (B′). In the presence of cisplatin ($< 10^{-8}$ M) the current intensity decreased linearly with cisplatin concentration for peak B′. Peak A′ also decreased, but in a non-linear fashion. When concentration of cisplatin was greater than 10^{-8} M, a third smaller peak at −0.68 V emerges. The decrease in peak B′ current was attributed to the inaccessibility of the disulfide linkages of HSA for reduction at the electrode surface, due to cisplatin binding, and the shoulder at −0.68 V could be ascribed to the reduction of the cisplatin–HSA binary complex, similarly to the interaction of cisplatin and cystine [35].

Trynda-Lemiesz and coworkers reported a comparative study of the effects of both isomers of diaminedichloroplatinum(II) (*cis* and *trans*) and the phosphonate derivative DBP (*cis*-diamine[(bis(phosphonatomethyl)amino)acetato(2-)-*O′N′*]platinum(II), Fig. 1) on the structure of commercial HSA [36] (Table 1). HSA binding to heme and bilirubin was decreased by cisplatin (10 and 65%, respectively); transplatin affected heme-binding sites to a larger extent (33%). On the other hand, DBP showed little interference with the protein structure and ligand-binding ability, probably due to slow kinetics of substitution of the phosphonate ligand.

Aggregation of albumin induced by cisplatin has been observed even at low cisplatin:rHA ratios (K_2PtCl_4 induced almost complete HSA dimerization at molar ratios of ca. 10:1) [37]. Dimers and higher cross-linked forms of albumin will be formed depending upon the complex:protein ratios and do not efficiently deliver the metal drug to the tumor targets. This could be an explanation for the contrasting results obtained during the studies of the biological activity of cisplatin–albumin adducts (experiments with 1:1 ratios were more successful than others with higher molar ratios; see [27] and references). In accordance with previous works by Ohta and colleagues [31], it was found that transplatin induced cross-linkage of HSA to a larger extent than the *cis* isomer [36]. DBP promoted no aggregation of the protein. The proposed dimer should be of the type *trans*-$(HSA)_2Pt$; transplatin could easily

labilize its two chloride ligands to result in this structure. Dimer formation starting from cisplatin, however, would imply in an initial cleavage of one cisplatin–NH_3 bond [36].

In aqueous media, cisplatin can undergo stepwise hydrolysis depending on the concentrations of the platinum complex and chloride ions. LeRoy and Thompson [38] found that the reaction of cisplatin with bovine serum albumin (BSA) at 37 °C proceeds via two first-order processes, initially through the aquation of the metal complex ($k = 0.35\ h^{-1}$; $t_{1/2} = 2.0$ h), this rate being equal to the rate for the direct reaction of uncharged cisplatin with the protein. The charged intermediate will react rapidly with the protein ($k = 1.80\ h^{-1}$; $t_{1/2} = 0.38$ h). Ormaplatin (Fig. 4), however, does not require the aquation step ($t_{1/2} > 30$ h) to react with bovine albumin. The direct first-order reaction of ormaplatin was slightly faster than that for cisplatin ($k = 0.42\ h^{-1}$; $t_{1/2} = 1.65$ h).

Nagai and coworkers [39] studied the kinetics of cisplatin with albumin in excess chloride, attaining a model closer to the actual biological systems. It was found that cisplatin binding to the protein obeyed a S_N2 mechanism previously described for the interaction of square-planar platinum(II) complexes with nucleophiles (Fig. 3). The apparent pseudo-first-order rate constant k_{app} correlated linearly with [albumin] (Table 2) and was given by

$$k_{app} = 0.263 + 0.405[\text{albumin}]$$

The complex *meso*-1-$PtCl_2$ (Fig. 1*j*; L = L′ = Cl) has been studied under the same mechanistic scope [43] but in contradiction with cisplatin, the direct binding of the complex with the protein did not account significantly for the overall rate constant. Table 2 gives some comparative data.

Also, reaction of *meso*-1-$PtCl_2$ with human plasma at 37 °C is very fast and >99% reversible. This reaction proceeds in two steps: formation of *meso*-1-$PtCl_2$–protein ($t_{1/2} \sim 35$ min) and subsequent release of the biologically active amine ($t_{1/2} \sim 70$ min). Binding of tritiaded *meso*-1-$PtCl_2$ with BSA at 0 °C ($t_{1/2} \sim 150$ min) prevents the initial formation of covalent bonds

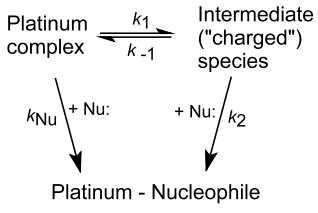


Fig. 3. Two-path mechanism for the S_N2 reaction of square-planar Pt(II) complexes. k_1 is the platinum complex hydrolysis rate constant, k_{Nu} is the second-order rate constant for the direct reaction of uncharged platinum complexes and nucleophile, k_2 is the rate constant for the reaction of hydrolyzed platinum species and the protein and Nu is a nucleophilic species (in this case, albumin) [39,40].

between the complex and albumin and is reversed in a concentration-dependent manner by the 'cold' *meso*-1-$PtCl_2$ but not by the organic ligand, which suggests a specific binding site in the protein. The rate of the reaction of *meso*-1-$PtCl_2$ with BSA at 37 °C is comparable to the rate of complex hydrolysis ($k = 0.27\ h^{-1}$), which would be the rate-determining step. Precluding hydrolysis by means of excess chloride slows the reaction, indicating that the hydrolysis product of *meso*-1-$PtCl_2$ is the reactive species (Table 2). It is interesting to notice that *meso*-1-$PtCl_2$ is more rapidly cleared from the plasma ($t_{1/2} \sim 35$ min) than cisplatin ($t_{1/2} \sim 100$ min), this difference being explained in terms of more significant globulin binding ($t_{1/2} = 22$–41 min) in the case of *meso*-1-$PtCl_2$ [40].

The complex *rac*-4F-Pt(CBDC) is an active ethylenediamine derivative of carboplatin (Fig. 1) and its interaction with HSA was studied by means of ultracentrifugation. The bidentate ligand cyclobutane-1,1-dicarboxylate renders this complex and carboplatin less prone to bionucleophilic attack, when compared with the analogous dichloride complex (*rac*-4F-$PtCl_2$) or cisplatin [41]. The complex *meso*-1-$PtSO_4$ (Fig. 1*j*; L = H_2O and L′ = SO_4) can rapidly exchange its sulfate anion with chloride. A comparison of the binding of this complex to HSA in vitro and in vivo with that of cisplatin showed that *meso*-1-$PtCl_2$ reacts faster ($t_{1/2} \sim 0.5$ and 3 h for *meso*-1-$PtCl_2$ and cisplatin, respectively [42]) and to a larger extent with HSA due to a stronger hydrophobic interaction. This protein-bound complex is believed to be unavailable for therapeutic purposes [43]. It is interesting to bear in mind that the reactions of platinum compounds with albumins usually take place after several minutes to hours; metallodrugs that react mainly through coordinate bonds such as those based on ruthenium and rhodium show a more rapid binding (see below).

As far as the biological importance of albumin–cisplatin is concerned, despite suggestions that this rather irreversible adduct would not constitute a drug reservoir for therapeutic purposes (see for example [44]), a number of reports show evidence of positive clinical effects of cisplatin–HSA: similar effects of both free and protein-bound cisplatin in seven tumor models [45]; increased tumor concentration of Pt after cisplatin–HSA administration [46]; and complete remission of laryngeal carcinoma after treatment with three courses of the adduct [47]. Albumin was found to enhance the chemotherapeutic effect of carboplatin [48]. A patent exists for the use of albumin- or transferrin-bound chemotherapeutics (including cisplatin) linked by functionalized organic ligands [49].

Also, endogenous thiols as glutathione may effectively compete with albumin for platinum-drug binding, as was shown by the system cisplatin–BSA [50] and a series of active 4′-chloro-2,2′:6′,2″-terpyridine platinum(II)

Table 2
Comparative kinetic constants for the first-order reactions of some platinum(II) compounds with albumins (see Fig. 3)

Cisplatin [39]		*meso*-1-$PtCl_2$ [40]	
[albumin] (g l^{-1})	k_{app} (h^{-1})	[albumin] (g l^{-1})	k_{app} (h^{-1})
10	0.317	6	0.28
20	0.509	30	0.24
50	0.579	40	0.23
100	0.817	40 + 100 mM Cl^-	0.19
		60	0.24
Obs. $k_1 = 0.263\ h^{-1}$; $k_{Nu} = 0.405\ M^{-1}\ h^{-1}$, [Pt] = 30 μM; [$Cl^-$] = 14 mM, 37 °C, pH 7.0		Obs. BSA; $k_1 = 0.27\ h^{-1}$, 37 °C, pH 7.0	

derivatives with rHA [51] in vitro. Although not fast, this transfer of platinum between thiol groups could explain the role played by antitumoral Pt compounds in the inhibition of some cell-cycle regulatory enzymes such as thioredoxin reductase, as well as other non-DNA targets [51].

Decrease in plasma albumin levels increase marrow, nefro-, hepato- or oto-toxicity of cisplatin, as well as the drug levels in pregnant women and fetus [52–55]. The response of hypoalbuminemic patients to cisplatin therapy is poor [56,57]. Surprisingly, however, analbuminemic rats treated with cisplatin showed no marked difference either in body clearance of Pt or renal function as compared with normal animals. Globulins could be responsible for attenuate possible harmful effects of cisplatin in the absence of albumin [58].

Albumin microspheres have been used for the controlled release of cisplatin, and both in vivo and in vitro properties and antitumor activity have been assayed, with or without emulsion stabilizing agents. This technique of drug delivery proved able to attenuate toxic side effects of cisplatin alone [59–64].

Other second-generation antitumor platinum(II) complexes (Fig. 1) have had their pharmacokinetics studied in different animal models. Oxaliplatin (1-OHP, Eloxatine) exhibited similar [65] or greater protein binding ability than cisplatin [66] or carboplatin [67], being similar to that of ormaplatin (Fig. 4b) [65]. In human plasma, γ-globulins and albumin shared similar levels of oxaliplatin within 3 h after i.v. administration [68]. Equilibrium between oxaliplatin and albumin alone or total plasma was attained after 24 and 5–6 h, respectively, being 79–87% of the Pt covalently bound to purified albumin [69]. However, other complexes with potential to clinical trials such as nedaplatin (254-S; glycolato-*O*,*O*′)diammine platinum(II)) [70] and lobaplatin [71] showed poor association with plasma proteins. In the other hand, active Pt(II) dimers like $[Pt_2(EtUra)_2(NH_3)_4](NO_3)_2$ (EtUra = ethyluracyl) and $[Pt_2(NaphCH_2Ura)_2(NH_3)_4](NO_3)_2$ ($NaphCH_2Ura$ = naphtylmethyluracyl) (Fig. 1) were found to bind extensively to BSA and other plasma proteins. Their

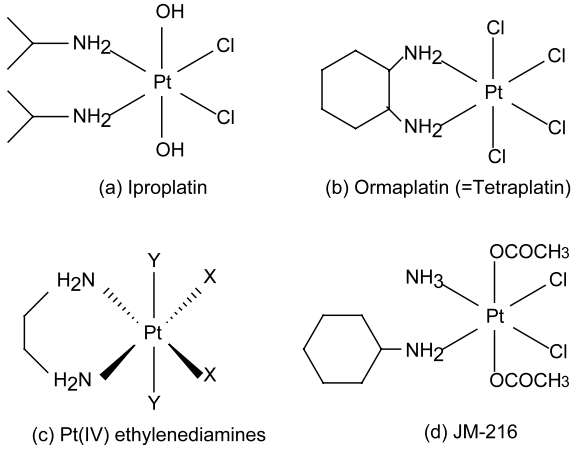


Fig. 4. Structure of some antitumoral Pt(IV) complexes.

cytotoxicity could be a result of either direct accumulation of non-bound complex or slow in vivo formation of cisplatin [72].

2.2. Pt(IV) complexes

The interactions of the ethylenediamine analogues of cisplatin *trans*,*cis*-[Pt(en)$(OH)_2I_2$] (Fig. 4c; X = I, Y = OH), *trans*,*cis*-[Pt(en)$(OH)_2Cl_2$] (Fig. 4c; X = Cl, Y = OH), [Pt(en)I_2] and [Pt(en)Cl_2] with rHA have been studied by means of gel filtration chromatography, determinations of the extent of thiol blocking, and UV–vis (Pt(IV)–I LMCT band), ICP-MS and 2D [^{1}H,^{15}N]-NMR spectroscopies [73].

These studies showed that the single free thiol at Cys34 is crucial for the reaction of *trans*,*cis*-[Pt(en)$(OH)_2I_2$] with HSA but not for the reaction with its chloro analogues or the Pt(II) diamines. Iodide from *trans*,*cis*-[Pt(en)$(OH)_2I_2$] can be exchanged for chloride ions in chloride-containing media, and the mono- and bi-substituted analogues can bind to the protein. Unexpectedly, it was found that [Pt(en)I_2] complex reacted faster with albumin than *trans*,*cis*-[Pt(en)$(OH)_2I_2$] or [Pt(en)Cl_2]. A mechanism was proposed (Fig. 5) in

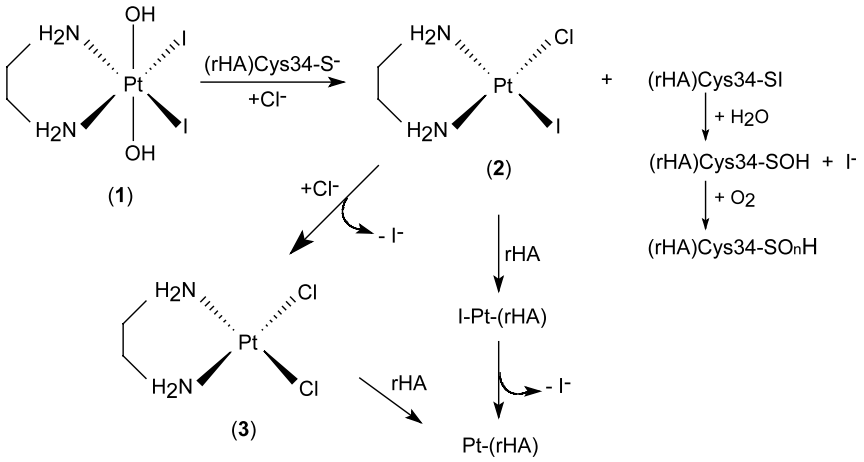


Fig. 5. Proposed mechanism for the reduction of *trans*,*cis*-[Pt(en)(OH)$_2$I$_2$] by recombinant human albumin. Initially, Cys34 attacks an iodide ligand (**1**), giving rise to the sulfenyl iodide, which then hydrolyzes to sulfenic acid, together with the monoiodo monochloro Pt(II) complex (**2**). This complex can undergo further substitution reactions, giving rise to **3** and adducts with the protein at sites other than Cys34. Further oxidation of the sulfenic acid group is irreversible. Reprinted with permission from reference [73]. ©1999, American Chemical Society.

which the iodide ligands are the first targets of the free thiol and, after reduction, platination occurs in sites other than Cys34 [73].

In accordance with this scheme, ICP-MS studies show that the two iodide ligands from *trans*,*cis*-[Pt(en)(OH)$_2$I$_2$] or [Pt(en)I$_2$] are released from the complex in separate stages, this reaction being fast in the first 2.5 h [74].

Studying the structure-activity of a series of Pt(IV)-ethylenediamines with different anionic ligands (Fig. 4c: X = Cl; Y = Cl, OH, $OCOCH_3$; X = I; Y = Cl, OH, $OCOCH_3$, $OCOCF_3$, OSO_2CH_3), Kratochwil and Bednarski [75] observed that the second-order rate constant of reduction of *trans*,*cis*-[Pt(en)(OH)$_2$I$_2$] ($k = 12.72$ M^{-1} s^{-1}) by BSA to its Pt(II) analogue was larger than that of the new sulfonate complex *trans*,*cis*-[Pt(en)$(OSO_2CH_3)_2$I$_2$]] ($k = 8.18$ M^{-1} s^{-1}). Glutathione showed a similar trend but no difference was observed when L-cysteine was the reductant. This observation indicated that the chemical environment of the thiol group is important in determining the reduction kinetics of Pt(IV) complexes. Even considering only the fraction of BSA that contains a free thiol group (Cys34), albumin is considerably less effective than L-Cys and GSH as a reducing agent. Reduction potentials of these complexes alone can not be used to predict whether a Pt(IV) complex will be reduced by biological thiols at physiological concentrations. The rate constant of reduction was also obtained for the reaction of *trans*,*cis*-[Pt(en)(OAc)$_2$I$_2$] with BSA ($k = 330.31$ M^{-1} s^{-1}), and ormaplatin reduction by BSA ($k = 112$ M^{-1} s^{-1}) was also slower than that by glutathione or cysteine [19], possibly reflecting difficulty in accessibility of Cys residues.

In vitro platinum binding to human plasma assessed through two ultrafiltration systems showed that iproplatin (Fig. 4a) presents no binding to plasma proteins [76,77]. After 24 h, the percentage of protein binding was 0, 31, 83, 89 and 94% for iproplatin, carboplatin, JM-40 (ethylenediamminemalonatoplatinum(II)), spiroplatin (aqua(1,1-bis(aminomethyl)cyclohexane)sulfatoplatinum(II)) and cisplatin, respectively [78]. Equilibrium dialysis experiments showed that the irreversibility of platinum binding to whole plasma proteins follows the order iproplatin (more reversible) < carboplatin < cisplatin < transplatin [79]. However, other authors did find 30% of ^{195m}Pt-labelled iproplatin binding to mouse plasma after 24 h [80].

Pharmacokinetic experiments in humans showed that ormaplatin exhibits a high degree of protein binding [81], albumin probably being the main biological target. A fast protein binding was reported of ormaplatin in tissue culture medium [19] as well as in the whole human ($t_{1/2} = 0.875$ h [19]) or mouse ($t_{1/2} = 2$ h [18]) plasma which correlated with the amount of free sulfhydryl, the disparity in the $t_{1/2}$ values being probably a result of different experimental setups. The importance of diaminecyclohexane release to the protein binding has been subject of some controversy (for a discussion, see [40]).

The orally-active complex JM-216 (Fig. 4d) does not bind to plasma proteins and to albumin in particular ($t_{1/2} > 24$ h), but its metabolite JM-118, a platinum(II) compound, binds faster ($t_{1/2} = 4.2$ h), resembling cisplatin ($t_{1/2} = 3.2$ h). Other identified JM-216 platinum(IV) metabolites like JM-383 and JM-518 will not react with albumin or globulins ($t_{1/2} > 24$ h). Also, JM-216 cannot be found in human plasma but is present in mouse plasma 1 h after administration, suggesting different metabolic pathways [82].

3. Gold compounds

3.1. Au(I) complexes

The licensed second-generation gold drug auranofin (Ridaura™), triethylphosphine(2,3,4,6-tetra-*O*-acetyl-glucopyranosato-*S*-)gold(I) or $Et_3PAuSATg$ in short (Fig. 6), originally employed in the treatment of arthritis, is cytotoxic and active against P388 leukemia and rapidly binds to the Cys34 residue of HSA. Its biological action is believed to be due to the release of phosphine to the tumor cell. The metal plays a role in protecting the phosphine against oxidation (which renders it non-toxic), and the phosphine stabilizes the metal and provides a transport mechanism through the lipophilic cell membrane. Changing the metal center does alter the antitumoral activity [20]. Plasma protein (especially albumin) binding of auranofin lowers, but not prevents its cytotoxic action and extent of cell association [83].

The interactions of auranofin with bovine and HSA have been extensively studied by means of chromatography and ^{1}H-, ^{31}P-NMR, ESR, Mössbauer and EXAFS spectroscopies, both with the pure proteins and in the whole blood plasma [21,84–87]. Differently from cisplatin, there is little doubt on the primordial role of Cys34 of HSA as the major binding site for Au(I) complexes, but this does not preclude the existence of other sites of lower affinity. An overall mechanism of the interactions between $R_3PAuSATg$ (R = Et or *i*Pr) and albumin has been proposed (Fig. 7).

Briefly, the mechanism can be described in four steps:

1) Fast binding of auranofin or its isopropyl derivative to a free thiol (Cys34).
2) The tetraacetylthioglucose (AtgSH) ligand displaces the phosphine when R = Et. The easiness of displacement of the phosphine (R_3P) increases from R = isopropyl to methyl or ethyl. So, iPr_3PAu–S–Alb (Fig. 7b) requires CN^- to initiate the reaction. Cyanide has higher affinity for gold than thiolates or phosphines. For a given phosphine, the ease of thiol displacement follows the order tetra-acetylthioglucose > 1-thioglucose > glutathione > ergothionine.
3) Et_3P is oxidized by one of the 17 disulfide bridges with participation of a water molecule. The bulky iPr_3 substituents hamper the water attack of P, leading to a long-lived triisopropylphosphonium thiolate, which decays to $iPr_3P{=}S$ (Fig. 7b), and traces of $iPr_3P{=}O$.
4) Further reactions of the Cys34-bound gold phosphine with $R_3PAuSATg$, as detected through ^{31}P-NMR, are not observed. SAtg does not dissociate from gold; however, Et_3PAu^+ formed from Et_3-PAuCl will react with Cys34 to form a S-bridged digoldphosphine and, provided enough excess, will lead to reactions with the imidazole nitrogens of His residues ([84] and references therein).

Penefsky spun column chromatography, which consists in a gel filtration chromatography accelerated by centrifugal force, allowed the kinetic study of the fast binding of auranofin with BSA [88]. In vivo, the reaction is second-order and its rate = k [AlbSH] [auranofin] (k has been estimated as $8 \pm 2 \times 10^2$ M^{-1} s^{-1}). At clinically relevant conditions (auranofin = 10–25 μM and mercaptalbumin ~400 μM) the reaction is pseudo-first order in auranofin and $t_{1/2} \sim 2$ s; thus auranofin is short-lived in the bloodstream. The rate determining step should be the albumin crevice-opening and exposure of Cys34 (Fig. 8), as detected through changes in the His3 Hε1 and Hδ2 resonances in vitro with BSA or whole human plasma [86,87]. This change in albumin conformation could explain the Trp fluorescence quenching of ca. 12% of HSA after reaction with Et_3PAuCl [89].

Studies have been performed on the reactions of albumin with gold metabolites such as gold–glutathione

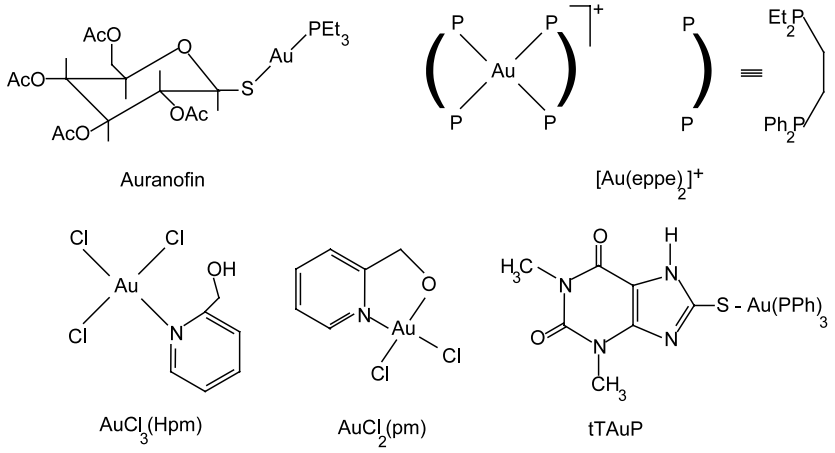


Fig. 6. Structure of antitumoral Au complexes.

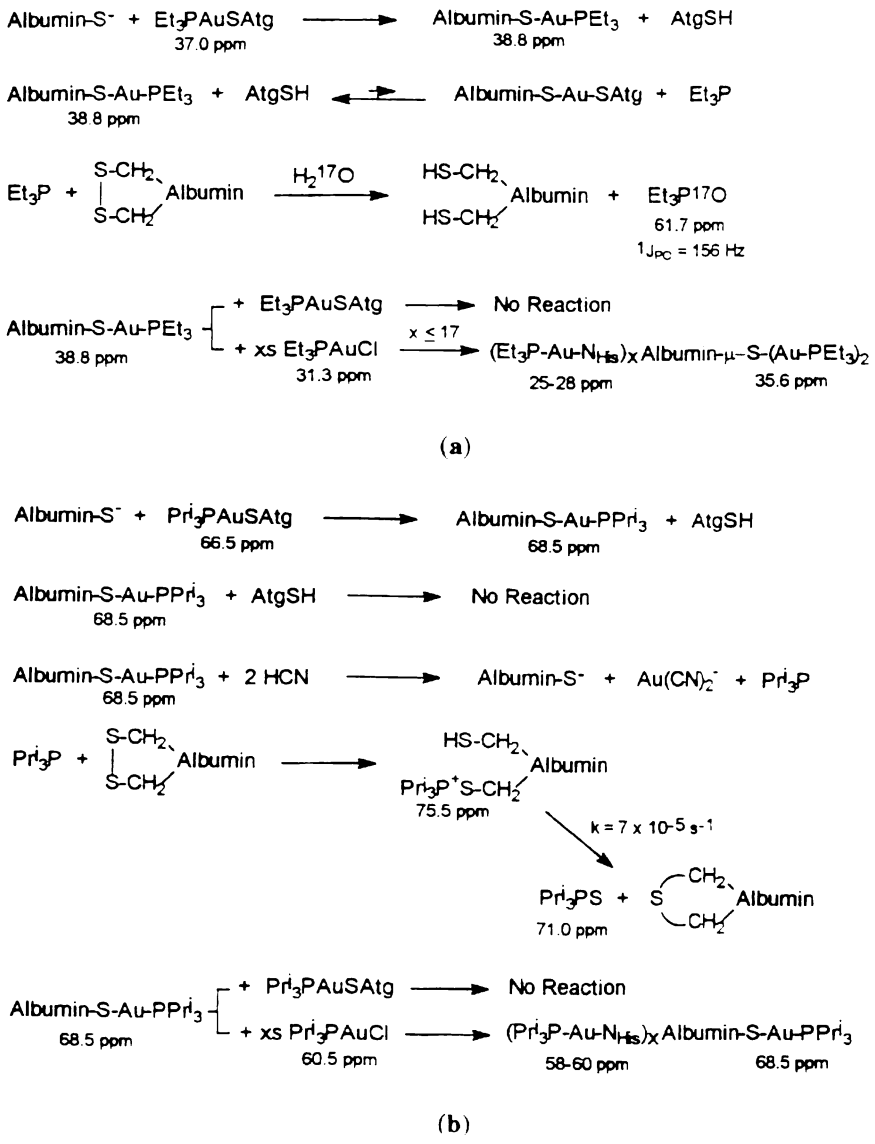


Fig. 7. Reactions of serum albumin with auranofin and auranofin analogues: (a) Et_3PAuX complexes; (b) iPr_3PAuX complexes (see text for further details). Reactions were conducted in buffered aqueous solutions at pH values near physiological pH. Reprinted with permission from reference [84]. ©1999, American Chemical Society.

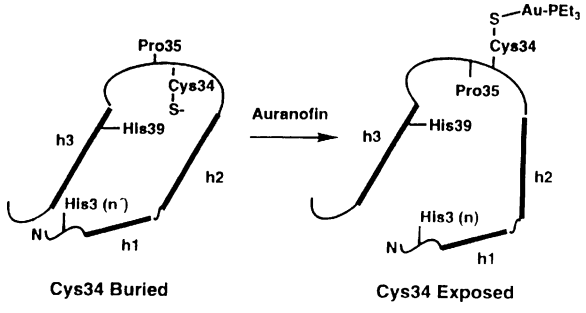


Fig. 8. Model for structural changes in domain IA of albumin. The Cys34 residue is in a crevice. It is proposed that gold binding (shown), or oxidation to form a disulfide at Cys34, or a sulfenic acid, leads to the movement of Cys34 to an exposed environment. This appears to be coupled to a movement of His3 in the N-terminal binding site of albumin. Reprinted with permission from reference [86]. ©1995, Federation of European Biochemical Societies.

[90] and $Au(CN)_2^-$ (aurocyanide, [91–93]). Contrary to auranofin and other gold compounds, aurocyanide does not react predominantly through Cys34 ($\leq 11\%$). Four binding sites have been found, one with $K = 5.5 \times 10^4$ and three others with $K = 7.0 \times 10^3$. These bound ligands are labile and could render the complex more bioavailable than other gold(I) complexes. Gold metabolites such as $AuPEt_3$ associated with albumin can be detected to the limit of 0.03 $\mu g\, l^{-1}$ by the direct injection nebulizer coupled to ICP-MS technique [94].

Thiols like 2-mercaptoethanol or dithiothreitol can compete for the gold, suggesting that this dissociation, at the membrane or inside the cell, could be thiol-assisted [95]. Like auranofin, other antitumoral gold(I) compounds have also activities sensitive to the presence of albumin: the diphosphine $[Au(eppe)_2]^+$, which reacts slowly with BSA [96], and the (8-thiotheophyllinate)(tri-

phenylphosphine)gold(I) (tTAuP, Fig. 6), whose cytotoxicity against Friend leukemia is significantly reduced in the presence of albumin (cell growth in the presence of 10 μM tTAuP is 1.6 and 46.0% increased as compared with the control when [albumin] = 0.2 and 20 mg ml^{-1}, respectively) [97].

3.2. Au(III) complexes

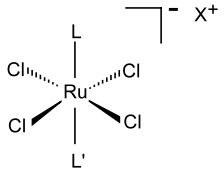
In spite of forming compounds similar to Pt(II) complexes, square-planar d^8 Au(III) complexes have been infrequently studied as alternatives for platinum-based chemotherapy [20,21,98].

The interactions of $AuCl_3$(Hpm) and $AuCl_2$(pm) (Fig. 6) with BSA have been initially studied by means of UV and circular dichroism (CD) spectroscopies [98]. Upon binding, both complexes present quick reduction from Au(III) to Au(I), as inferred through the disappearance of the LMCT gold(III) band at 315 nm in a few minutes. This reduction was tentatively attributed to gold(III) binding to Cys34. At a 1:1 complex to protein ratio, there are no meaningful differences in the CD spectra of the protein, indicating that the binding is not specific.

The bifunctional Au(III) complex with the cytotoxic quinone antibiotic streptonigrin was designed to allow the controlled release of the toxic organic molecule as Au(III) was reduced by biothiols. It is a very stable complex, presenting no evidence of interaction with albumin at a 1:10 complex to protein ratio after several hours [99].

4. Ruthenium(III) compounds

The reaction of imidazolium [*trans*-tetrachlorobis(imidazol)ruthenate(III)], HIm-*trans*[$RuCl_4(Im)_2$] (Fig. 9) with HSA was followed by spectroscopic (Table 1) and immunological methods [100]. The complex performs two hydrolytic steps. Within 30 min the mono-hydroxy-Ru(III) complex binds to the protein giving rise to two positive CD transitions at 340 and 430 nm with a shoulder around 520 nm, characteristics of bidentade binding to the protein. After 24 h the band at 340 nm decreases in intensity at the same time that an intense band around 530 nm appears, which corresponds to the second hydrolytical step. Protein fluorescence quenching was possibly a result of binding at His242 or at other His residues close to the Trp214 residue, which would bring it to a more solvent-exposed environment. Further alterations in the protein structure caused by HIm-*trans*[$RuCl_4(Im)_2$] binding were confirmed by the study of probe binding to ruthenium-HSA adducts. At equimolar ratios, only the strongest Ru-binding sites will be occupied. Heme and warfarin binding decreased (10 and 50% of the control, respectively) but bilirubin was relatively unaffected (90% of the control). This observation suggests that the ruthenium compound could be binding in the region around Trp214 in the subdomain IIA of HSA (warfarin binding site) which would cause also the decreased heme binding. Antigenic studies were performed with HIm-*trans*[$RuCl_4(Im)_2$]:HSA (10:1); only 20% antigenicity due to unfolding of the antigenic structure was observed. This relatively low immune response is due to the fact that the main antigenic site of HSA is not located in subdomain IIA, in accordance with probe binding studies [100].



L = L' = Im; X = HIm	HIm-*trans*[$RuCl_4(Im)_2$]
L = Im; L' = DMSO; X = Na	Na-*trans*[$RuCl_4(Im)(DMSO)$]
L = L' = Im; X = HIm	HIm-*trans*[$RuCl_4(Im)_2$]
L = L' = Ind; X = HInd	HInd-*trans*[$RuCl_4(Ind)_2$]
L = L' = Ind; X = Na	Na-*trans*[$RuCl_4(Im)_2$]
L = L' = DMSO; X = Na	Na-*trans*[$RuCl_4(DMSO)_2$]

Fig. 9. Ru(III) indazole (Ind) or imidazole (Im) complexes. HInd and HIm correspond to the protonated forms of indazole and imidazole, respectively.

Binding of HIm-*trans*[$RuCl_4(Im)_2$] and the indazolium analogues HInd-*trans*[$RuCl_4(Ind)_2$] and Na[$RuCl_4(Ind)_2$] (Fig. 9) with HSA were studied by means of size-exclusion chromatography coupled to ICP-MS [101] as well as by UV–vis, CD, fluorescence and ICP-AES spectroscopies [102]. Na[$RuCl_4(Ind)_2$] binding to this protein as a function of time was reported, but no kinetic parameters were given. HInd-*trans*[$RuCl_4(Ind)_2$] is insoluble in buffer, giving a blue-green precipitate, which can be dissolved with ethanol, the final solution showing an absorption peak at 585 nm. In presence of HSA this band remains but no precipitate is formed, indicating that the indazolium ruthenium complex is bound to the protein. Low-pressure liquid chromatography of the reaction products of this complex with both whole plasma and a mixture of albumin and apotransferrin (10:1) showed that 80–90% of HInd-*trans*[$RuCl_4(Ind)_2$] is albumin-bound. The reaction proceeds quickly with isolated proteins ($\sim$4–5 min) [8,103]. Four equivalents of HInd-*trans*[$RuCl_4(Ind)_2$] were found to bind per mol of HSA, putatively through His residues close to the Trp214 residue (which would account for the pronounced fluorescence quenching, Table 1) and in the proximity of the warfarin binding site. In addition, heme and bilirubin binding to the protein was disturbed by the presence of the ruthenium complex [102].

The interaction of another imidazolium derivative of ruthenium, Na-*trans*[$RuCl_4(Im)(DMSO)$] (Fig. 9) with

BSA was also studied [104]. This complex undergoes stepwise hydrolysis in solution, the first of which is accelerated by the protein. The first Cl^- loss gives rise to a band at 346 nm characteristic of $[Ru(OH)Cl_3(Im)(DMSO)]^-$. BSA did not reduce the metal, as indicated by the permanence of the band at 400 nm characteristic of $[RuCl_4(Im)(DMSO)]^-$. Up to five equivalents of Na-*trans*$[RuCl_4(Im)(DMSO)]$, Na-*trans*$[RuCl_4(DMSO)_2]$, HIm-*trans*$[RuCl_4(Im)_2]$ [104] or HInd-*trans*$[RuCl_4(Ind)_2]$ [103] are bound to the albumin molecule. The first equivalent of Na-*trans*$[RuCl_4(Im)(DMSO)]$ binds to BSA with high specificity, giving rise to a distinct positive CD transition at 410 nm. Further complex molecules show rather unspecific binding over the surface of the protein. Histidine imidazols have been suggested as the preferred binding sites, since ruthenated BSA is less reactive to diethylpyrocarbonate, a common modifier of these residues [104].

The polyaminocarboxylate derivative $[Ru(PDTA)Cl_2]^-$, dichloro 1,2-propylendiaminetraacetate ruthenium(III) was reacted with BSA at a molar ratio of 1:1 [105]. The reaction proceeded via loss of at least one Cl^- ligand. Surprisingly, visible CD did not reveal any bands attributable to the metal complex, a behavior different from that of the imidazolium derivatives discussed. This complex, however, possesses specific hyperfine ^{1}H-NMR patterns, which are not detectable in ultrafiltrates, indicating that $>90\%$ of the ruthenium should be protein-bound. The patterns of proton NMR spectral displacements are similar for the reaction of $[Ru(PDTA)Cl_2]^-$ with BSA, human apotransferrin or human diferric transferrin, indicating that the protein-binding mode could be similar (to histidine residues for instance) but rather non-specific (absence of CD spectra changes).

5. Rhodium(II) compounds

Rhodium(II) complexes of general formula $Rh_2(bridge)_4$ (Fig. 10) are the most extensively studied rhodium-based antitumor systems. Bear and coworkers pioneered these studies, and in an early report showed through equilibrium dialysis that rhodium acetate (Fig. 10, bridge $= CH_3CO_2$) could bind to BSA and other proteins, but no further characterization of the adduct was performed [106]. Further experiments have been carried out by Trynda and Pruchnik [107], studying the interactions of $Rh_2(CH_3CO_2)_4$ with defatted HSA by means of CD and electronic spectroscopy and quantitative immunoprecipitation. Spectrophotometric titration showed that around eight equivalents of the rhodium complex are bound to the protein, probably at His residues through the labile axial positions, judging by the displacement of the Rh–Rh absorption band from ca. 580 to 545 nm, and in accordance with

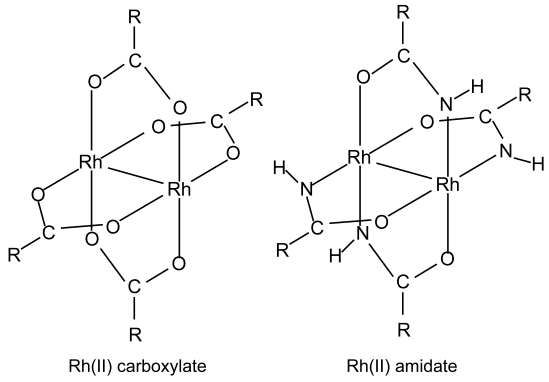


Fig. 10. Structure of Rh dimers.

previous works relating the axial, coordinative interaction of rhodium carboxylates with histidine peptides [108]. Defatted human albumin was quite sensitive to rhodium binding and its antigenic site was affected by this reaction (Table 1) [107].

These authors also studied the reaction of $[Rh_2(CH_3CO_2)_2(bpy)_2(H_2O)_2](CH_3CO_2)_4$ with defatted HSA. Similar structural alterations of the protein as well as fluorescence quenching were observed (Table 1), linked to decrease of binding of warfarin, heme and bilirubin. Again, after spectroscopic evidence (Rh–Rh band displaced to 470 nm after protein binding) the putative binding site was suggested to involve a His residue. Six to seven complex molecules were bound to the protein, according to gel chromatography studies [109].

These investigations were extended to a series of antitumoral rhodium(II) complexes, the carboxylates $Rh_2(CH_3CO_2)_4$, $Rh_2(CH_3CH_2CO_2)_4$, $Rh_2(CH_3CH_2CH_2CO_2)_4$ and $Rh_2(CF_3CO_2)_4$ (respectively, rhodium acetate, propionate, butyrate and trifluoroacetate), and the trifluoroacetamidate $Rh_2(CF_3CONH)_4$. All these complexes bind quickly to albumin in whole plasma [110] or in vitro to non-defatted HSA at a molar ratio of ca. 8:1 as shown through spectrophotometric titration [111]. The suggested binding sites were exposed His residues. Decrease in α-helix content was poorly observed even after 24 h incubation at 37 °C with large excess of complex. In the case of the alkylcarboxylates, both denaturating action and Trp fluorescence quenching efficiency correlated with lipophilicity, rhodium butyrate exhibiting a marked disturbance over the polypeptide (Table 1). Curiously, it is the amphiphilic complex $Rh_2(CF_3CO_2)_4$, which causes the largest losses in helicity and Trp fluorescence intensity. The rhodium center in this complex is a harder acid due to the electron-withdrawing effect of the CF_3 groups, which could favor its axial interaction with basic residues of HSA and/or with charged side chains of the peptide framework. The complex $Rh_2(CF_3CONH)_4$ also induced little structural alteration in the protein, and its apparent quenching ability could have been masked by

the coincidence of the complex *d*,*d*-absorption with the emission maximum of HSA [111].

Binding constants of these complexes with HSA were determined spectrophotometrically as $K = 1683$, 1057, 500.5, 712.3 and 214.1 for rhodium acetate, propionate, butyrate, trifluoroacetate and trifluoroacetamidate, respectively. The observed negative trend of the binding affinity with lipophilicity suggested that the hydrophobic interactions of the equatorial bridging ligands with albumin could compete with axial coordination with protein imidazoles [110]. However, integrity of the metal complexes after binding to albumin was not determined, which could help to clarify the exact nature of the interaction. Rhodium(II) complexes also diffuse into intact Ehrlich cells in a lipophilic-dependent way, in both free or previously albumin-bound forms. The bioactive complex $Rh_2(CF_3CONH)_4$ was reacted with clinical grade HSA and the final adduct induced meaningful (ca. 20% higher) survival in Balb-c mice bearing Ehrlich ascites, showing that the albumin-coordinated complex is not completely unavailable for therapeutic purposes [110].

Acknowledgements

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