Tumor-inhibiting ruthenium complexes – formulation and analytical characterization

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Tumor-inhibiting ruthenium complexes are presented as potential anti-cancer drugs.Their galenic formulations are given and hydrolytic reactions are highlighted. Interactions of Ru(III) complexes with serum proteins and with oligo- and polynucleotides are discussed.

Some ruthenium complexes like "ruthenium red", $[(NH_3)_5Ru\text{-}O\text{-}(NH_3)_4Ru\text{-}O\text{-}Ru(NH_3)_5]Cl_6$, *cis*-$[Ru(NH_3)_4Cl_2]Cl$ and ruthenium-DMSO complexes, like *cis*- and *trans*-$[RuCl_2(DMSO)_4]$, which show in vivo antitumor activity in several murine models including a *Cisplatin*-resistant P388 leukemia line, or Na *trans*-$[RuCl_4(DMSO)im]$, which reduces metastasis formation, exhibit antitumor activity. In particular, complexes of the general formula HL *trans*-$[RuL_4Cl_2]$, in which L is a N-heterocycle like indazole (abbreviation used: ind) or imidazole (abbreviation used: im) e.g., are active in different antitumor screening systems [1,2]. Figure 1 shows the structures of the compounds imidazolium *trans*-[tetrachlorobis(imidazole) ruthenate(III)], HIm *trans*-$[RuCl_4(im)_2]$, and indazolium *trans*-[tetrachlorobis(indazole)ruthenate(III)], HInd *trans*-$[RuCl_4(ind)_2]$. These Ru(III)-complexes exhibited the best results in antitumor tests, as well as in an autochthonous colorectal carcinoma of rats, a model that resembles the colon cancer of humans in its histological appearance and its behavior against chemotherapeutics [3]. In comparison, the well established antitumor drug *Cisplatin*, *cis*-$[PtCl_2(NH_3)_2]$, is completely inactive in this model. If one is aware of the high percentage of cancer mortality caused by tumors of the colon and the fact that no satisfactory chemotherapy exists, it is clear that development of these ruthenium-based drugs is of outstanding importance.

Although many groups are working on platinum based antitumor drugs since the discovery of the tumor-inhibiting qualities of *Cisplatin* by Barnett Rosenberg in 1969, a conclusive mode of action has not been found as yet. Even less is known about non platinum antitumor drugs like Ru-complexes and their mode of action. It should be of special interest to enhance knowledge in that field as it might lead to a better understanding of the differences between these metal-based drugs concerning toxicity or selectivity for different tumors. Therefore galenic formulation, hydrolysis reactions, interaction with serum proteins and reactions taking place in

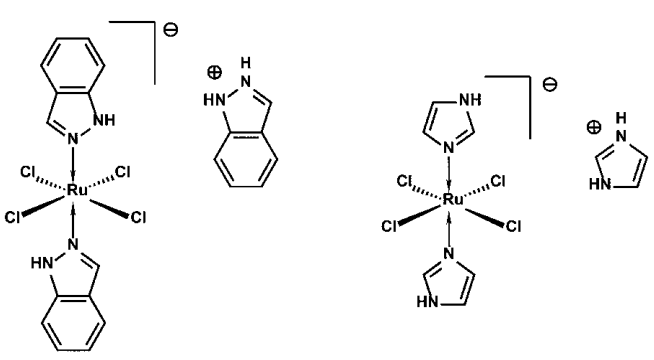

Figure 1. Tumor-inhibiting Ru(III)-complexes HInd *trans*–$[RuCl_4(ind)_2]$ (left) and HIm *trans*–$[RuCl_4(im)_2]$ (right).

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the cell (redox reactions, binding towards DNA, RNA and other occurring target molecules) have to be investigated. The obtained results could lead to new improved, "fine-tuned" drugs with enhanced activity and selectivity and reduced side effects.

Galenic formulation and hydrolysis reactions

HInd *trans*-[$RuCl_4(ind)_2$] is the most promising tumor-inhibiting Ru(III)-complex. To improve the poor water solubility of this compound, making a galenic formulation for clinical trials difficult, we synthesized the corresponding sodium salt Na *trans*-[$RuCl_4(ind)_2$] in a two step ion exchange via the tetramethylammonium salt. The sodium salt, obtained as a lyophilized powder, shows a 30 fold improved solubility in water compared to the original indazolium salt. As water has to be used as solvent for the last ion exchange reaction, hydrolysis reactions of the complex anion are a crucial point, limiting time scale of the preparation process.

The knowledge of the rate of hydrolysis and occurring hydrolysis products is also of interest with regard to storage and clinical application of this and other ruthenium complexes as well as to further reactions of the drugs in blood and cell.

Therefore we are investigating the hydrolysis of the Ru(III)-complexes under different conditions, like varying temperature, pH or NaCl concentration by means of UV/VIS, NMR-spectrometry, HPLC, pH- and conductivity measurements.

The complex salt HIm *trans*-[$RuCl_4(im)_2$] was already investigated in water and solvents like DMSO and ethanol by UV [4] and NMR [5]-spectroscopy. Aquation of the imidazole complex leads to mono- and diaqua complexes and eventually to a trisimidazole complex (reaction with the imidazolium counterion). Initial aquation to a monoaqua complex seems to play a crucial role for further reactions, since reaction with biological substrates is much faster in "aged" solutions of HIm *trans*-[$RuCl_4(im)_2$] than in "fresh" solutions

First investigations into the hydrolysis of HInd *trans*-[$RuCl_4(ind)_2$] suggest that hydrolysis proceeds slower but leads temporary also to aquacomplexes. The crystal structure of a monoaqua complex of the corresponding 1-methylindazole complex could be resolved [6]. As the formation of aqua complexes results in leaving chloride ions, chloride ion (physiological saline) concentration is another parameter to play a role in hydrolysis equilibria. Further hydrolysis products are unknown but could be µ-oxo-complexes. Formation of such di- or polynuclear Ru-complexes is pH dependent, with hydrolysis proceeding faster at higher pH, leading to precipitation. Figure 2 shows some possible hydrolysis reactions and decomposition compounds of the complex anion *trans*-[$RuCl_4(ind)_2$]$^-$

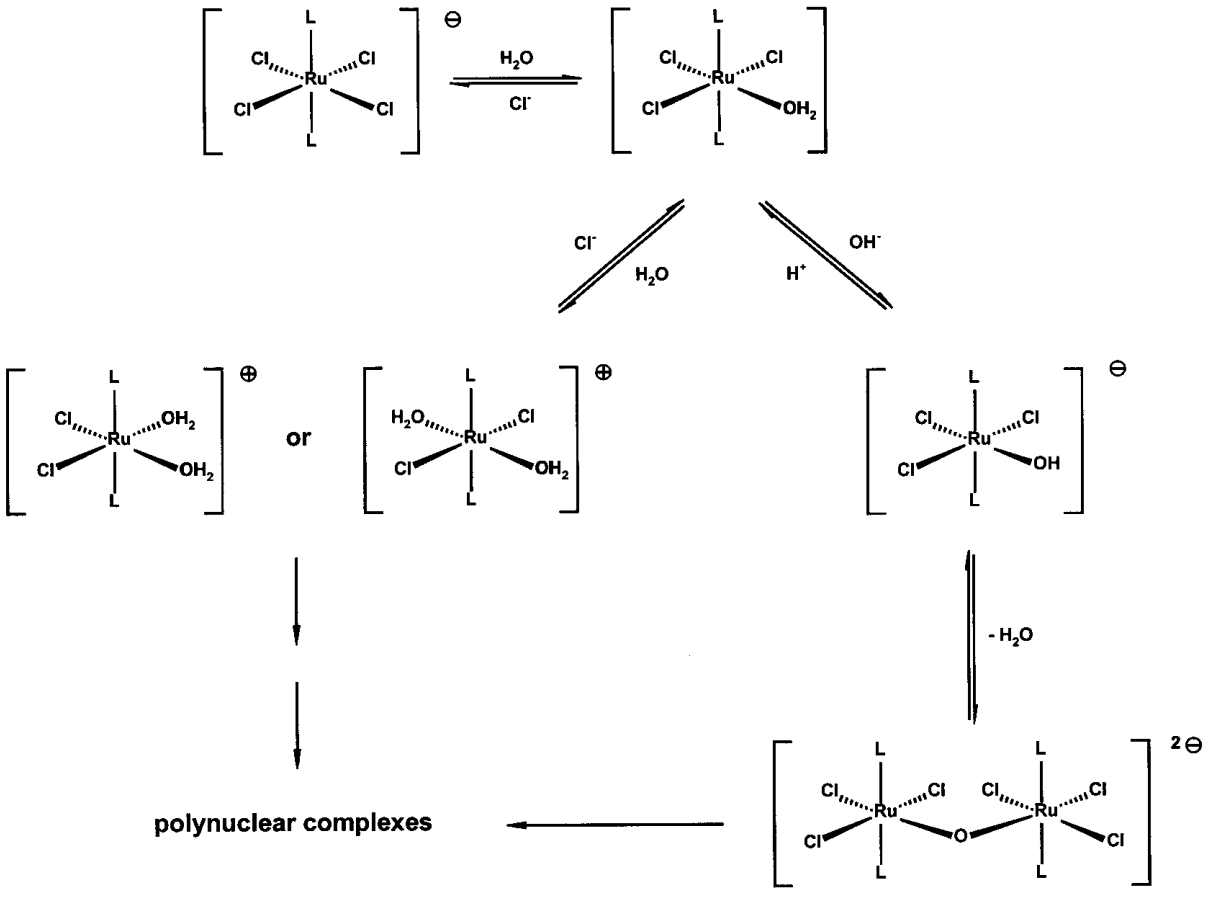

Figure 2. Possible hydrolysis pathways and decomposition species of the complex anion *trans*–[$RuCl_4L_2$]$^-$

Reversed phase chromatography can be useful in separating hydrolysis products, although one has to consider that the complexes or their hydrolytic successors can react not only with water but also with eluents like methanol or acetonitrile leading to solvolysis products not identical with those obtained in "pure" water or physiological buffer.

An analytical method that could be of special interest is coupling of HPLC with mass spectrometry. The crucial point in this approach would be ionization of the compounds, for example by ESI. One would have to deal with differently charged species like the original complex anion $[RuL_4Cl_2]^-$, the neutral monoaquacomplex $[RuL_4(H_2O)Cl]$ or the cationic diaquacomplex $[RuL_4(H_2O)_2]^+$ and with di- or polynuclear Ru-complexes.

Interaction of Ru(III) complexes with serum proteins

Interaction of drugs with serum proteins plays an important role in distribution of the drug in the body and affects features like toxicity and biological activity. Animal experiments in the autochthonous colon cancer model in rats have shown that HInd *trans*-$[RuCl_4(ind)_2]$ is far less toxic than HIm *trans*-$[RuCl_4(im)_2]$ but also exhibits a slightly higher antitumor activity. This difference in toxicity might be related to the different protein binding ability of the two compounds, assuming that the free complex is responsible for systemic toxicity. In comparison, over 90% of the platinum found in blood 3 – 4 h after administration of *Cispaltin* (25% in the case of *Carboplatin*) is bound irreversibly to plasma proteins, and the protein bound species have no significant antitumor activity and are not as toxic as *Cisplatin.* The loss of activity of *Cisplatin* when bound to plasma proteins is probably due to the irreversible binding to cysteines of plasma proteins such as albumin.

First investigations by means of CD spectroscopy and LPLC (UV detection) showed that Hind *trans*-$[RuCl_4(ind)_2]$ binds within a few minutes to the serum proteins albumin (M_r: 66.5 kDa) and transferrin (M_r: 80 kDa, responsible for iron transport) [7]. Interpretation of CD spectra suggests that apo-transferrin (the "iron-free" form of the protein) specifically binds two equivalents of HInd *trans*-$[RuCl_4(ind)_2]$. Albumin seems to bind five equivalents. X-ray crystallographic structure analysis of crystals of structurally transferrin-related human apo-lactoferrin that were treated (soaked) with solutions of HInd *trans*-$[RuCl_4(ind)_2]$ and HIm *trans*-$[RuCl_4(im)_2]$, was used to obtain binding sites for the Ru-complexes by difference Fourier analysis [8]. These investigations show that binding can not only specifically occur at the C- and N-lobe iron-binding cleft of the protein, but also at histidine residues on the surface of the protein (see Fig. 3).

It is assumed that the Ru-complexes can be transported to the tumor cell via the transferrin cycle. Because tumor cells have an increased requirement of iron, they have a higher number of transferrin receptors than normal cells. This enables accumulation of the Ru-complexes selectively in tumor cells (indirect drug targeting).

Thus, further investigations into protein binding of different Ru-complexes should focus on different serum proteins, like albumin and transferrin, as well as on separation of protein fractions of whole serum samples, incubated with Ru-compounds. For the latter, HPLC-methods using size exclusion, reversed phase, or ion exchange columns will be important.

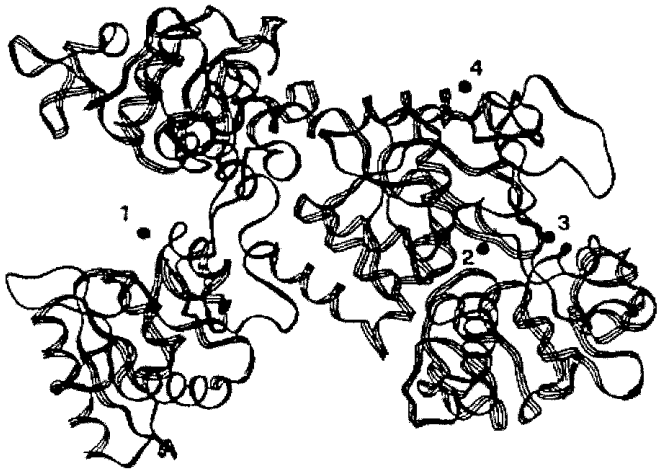

Figure 3. Ribbon diagram of apo-lactoferrin showing sites of HIm *trans*–$[RuCl_4(im)_2]$-binding after soaking for four weeks. Sites are: (1) His 253, (2) His 597, (3) His 590, (4) His 654.

Besides mode and rate of binding, release of ruthenium from the protein, especially from transferrin, is of interest, because the free, maybe structurally transformed, Ru-complex should be responsible for antitumor activity and further reactions in the tumor cell.

Binding of Ru-complexes to oligo- and polynucleotides

As in the case of platinum complexes, interaction of Ru-complexes with DNA is assumed to be responsible for antitumor activity, although other additional or supplementary mechanisms are possible as well.

Investigations into binding of HInd *trans*- $[RuCl_4(ind)_2]$ and HIm *trans*- $[RuCl_4(im)_2]$ towards calf-thymus DNA and the synthetic double-stranded homopolymers poly(dG) · poly(dC) and poly(dA) · poly(dT) were carried out using UV/VIS- and ICP-AES [9]. Both complexes bind covalently to calf-thymus DNA and show a binding preference for poly(dG) · poly(dC) compared to poly(dA) · poly(dT).

Further investigations are necessary to obtain a deeper insight into binding of the Ru-complexes to nucleobases and possible DNA intra- or interstrand cross linking properties. The use of NMR-techniques, helpful in the case of platinum complexes, is limited because of paramagnetism of Ru(III)-compounds.

Also of great importance will be the knowledge of redox reactions, taking place in the hypoxic milieu of the tumor cell, as the resulting Ru(II)-species should exhibit other ligand, nucleophile preferences than Ru(III)-complexes.

Thus, Ru(III)-complexes have to be seen as prodrugs, being transformed into antitumor active species in the body by hydrolysis and redox reactions and reactions with biologically occurring nucleophiles.

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