

Recent developments in ruthenium anticancer drugs

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Received 26th February 2009, Accepted 10th July 2009

First published as an Advance Article on the web 13th August 2009

DOI: 10.1039/b904071d

Interest in Ru anticancer drugs has been growing rapidly since NAMI-A ((ImH⁺)[Ru^{III}Cl₄(Im)(S-dmsso)], where Im = imidazole and S-dmsso = S-bound dimethylsulfoxide) or KP1019 ((IndH⁺)[Ru^{III}Cl₄(Ind)₂], where Ind = indazole) have successfully completed phase I clinical trials and an array of other Ru complexes have shown promise for future development. Herein, the recent literature is reviewed critically to ascertain likely mechanisms of action of Ru-based anticancer drugs, with the emphasis on their reactions with biological media. The most likely interactions of Ru complexes are with: (i) albumin and transferrin in blood plasma, the former serving as a Ru depot, and the latter possibly providing active transport of Ru into cells; (ii) collagens of the extracellular matrix and actins on the cell surface, which are likely to be involved in the specific anti-metastatic action of Ru complexes; (iii) regulatory enzymes within the cell membrane and/or in the cytoplasm; and (iv) DNA in the cell nucleus. Some types of Ru complexes can also promote the intracellular formation of free radical species, either through irradiation (photodynamic therapy), or through reactions with cellular reductants. The metabolic pathways involve competition among reduction, aquation, and hydrolysis in the extracellular medium; binding to transport proteins, the extracellular matrix, and cell-surface biomolecules; and diffusion into cells; with the extent to which individual drugs participate in various steps along these pathways being crucial factors in determining whether they are mainly anti-metastatic or cytotoxic. This diversity of modes of action of Ru anticancer drugs is also likely to enhance their anticancer activities and to reduce the potential for them to develop tumour resistance. New approaches to metabolic studies, such as X-ray absorption spectroscopy and X-ray fluorescence microscopy, are required to provide further mechanistic insights, which could lead to the rational design of improved Ru anticancer drugs.

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Cisplatin and its derivatives: a blueprint for the development of ruthenium anticancer drugs

A steady growth of interest in Ru anticancer drugs over the last 20 years is reflected in the accelerating growth of



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publications in this area (Fig. 1, based on a search in the *Chemical Abstracts* database).¹ The biological activities (including anticancer activities) of Ru complexes were first recognised by Dwyer and co-workers in the 1950s,^{2–4} but this research was largely forgotten until the serendipitous discovery of cisplatin by Rosenberg and co-workers in the 1960s,^{5–7} and the subsequent developments in Ru anticancer drugs often mirrored those of Pt drugs. Hence, it is logical to start with a brief overview of the currently accepted mechanism of anticancer activity of Pt complexes (Scheme 1).⁶

The cytotoxicity of cisplatin (*cis*-[Pt^{II}Cl₂(NH₃)₂], **1a** in Scheme 1) in common cancer cell lines is several orders of magnitude higher than that of transplatin (**1b** in Scheme 1),⁸ which shows that isomers with the same donor ligands can have quite different biological activities. Under physiological conditions, the relatively labile chlorido ligands (also called leaving groups) of **1a** are replaced within several hours by aqua/hydroxido ligands, which leads to partially hydrolysed species such as **2** (Scheme 1), which then readily bind to biological macromolecules.⁹ It is thought that in order to execute its cytotoxic function, a molecule of **1a** has to diffuse unchanged into the cell nucleus, where aquation and hydrolysis would occur, followed by DNA binding (**3** in Scheme 1).^{6,7} In some cases, binding of an aquation and/or hydrolysis product of **1a** to two adjacent guanine bases in the DNA chain can cause a severe distortion in the DNA structure (as illustrated in **3**), which is not repaired by the existing enzymatic mechanisms, and triggers cell death *via* apoptosis.^{6,7} Premature aquation and hydrolysis of **1a** in the extracellular medium leads to the formation of Pt adducts with proteins (primarily serum albumin, **4** in Scheme 1).¹⁰ This undesirable process is thought to deactivate up to 98% of **1a** administered by intravenous injection, despite the fact that the rate of aquation of **1a** in the blood plasma is lower than that in the cells due to the higher concentration of Cl⁻ ions (150 mM extracellular *versus* 4–20 mM intracellular),^{6,7,11} *i.e.*, Cl⁻ competes more effectively as a nucleophile than does H₂O or proteins at

the higher extracellular concentration of Cl⁻. Further deactivation of **1a** can occur in the cytoplasm due to its binding to intracellular S-donors, such as glutathione (binding of Pt(II) to S-donors is favoured due to the “soft” nature of both Pt and S atoms).^{6,11} It has to be emphasised, however, that alternative cytotoxicity mechanisms to that outlined in Scheme 1 are now seriously considered as contributing to the biological activities of these drugs. These mechanisms include the interactions of Pt complexes with the plasma membrane,¹² or with regulatory proteins.¹³ Generally, the rates of ligand-exchange reactions that are comparable with those of cell-division processes seem to be responsible for the anticancer activity of Pt(II) complexes.¹⁰

While Pt drugs are used extensively in oncology, there is a limited range of activity of cisplatin (mainly used in the treatment of testicular and ovarian cancers), although it has a much wider range of activity in combination therapy.¹⁴ The main issues with cisplatin, apart from its limited range of activity, are its high systemic toxicity¹⁵ and the propensity for patients to develop tumour resistance,¹⁶ all of which have led to considerable efforts to design alternative Pt anticancer drugs with lower toxicity, fewer issues with tumour resistance, and/or a broader spectrum of activity.^{14,17} As a result of synthesis and testing of thousands of drug candidates, only two more Pt(II) complexes (carboplatin and oxaliplatin, **5a** and **5b** in Scheme 1) have reached wide clinical use.^{6,7,18} The use of carboxylato instead of chlorido ligands as leaving groups (**5a** and **5b**), and the use of a more inert chelating N-donor ligands instead of NH₃ (**5b**) cause an increase in water solubility and favourable changes in the ligand-exchange reaction rates compared with **1a**.^{14,17,19} Much recent effort has also been directed in the last decade towards the design of orally active Pt(IV) compounds, such as satraplatin (**6** in Scheme 1), but all these compounds are still in the pre-clinical research stage.^{18–20} In contrast to square-planar Pt(II) complexes, **1a,b** or **5a,b**, octahedral Pt(IV) compounds, such as **6**, are stable for days in biological media, so that they can reach the tumour site unchanged, and then be gradually converted to more labile Pt(II) species by biological reductants



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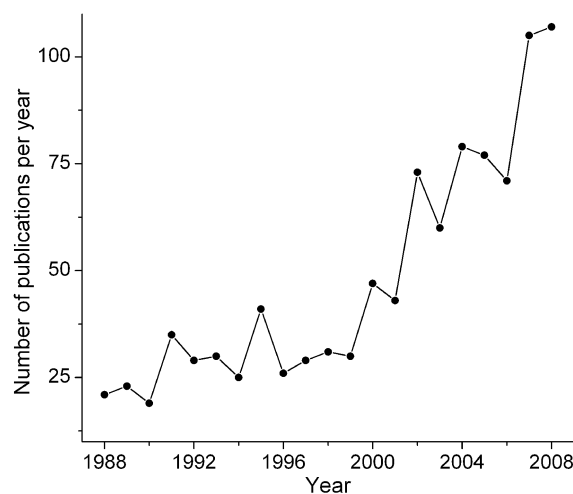
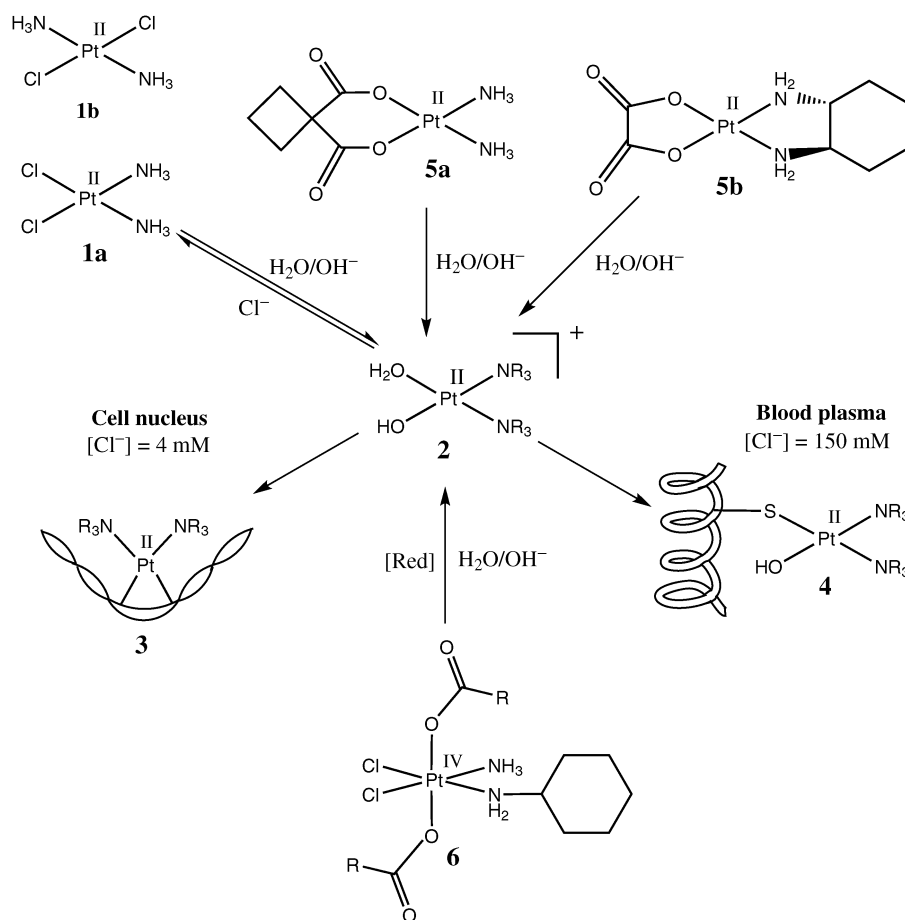


Fig. 1 Growth in publications on Ru anticancer drugs (searched in the *Chemical Abstracts* database).¹



Scheme 1 Commonly accepted mechanism of action of Pt anticancer drugs (based on the data from ref. 6).

(such as ascorbate, glutathione and NAD(P)H), assisted by the hypoxic environment that typically exists within solid tumours.²⁰ The accumulated experience in the development of platinum anticancer drugs provided the impetus to explore the more versatile ligand-exchange and redox properties of other Pt-group metals, including Ru.^{14,21,22}

Structures and activities of ruthenium anticancer drugs

Studies of medicinal applications of Ru compounds have been facilitated by the wide diversity of the coordination and organometallic chemistry of Ru that has been developed in fundamental research,^{23,24} as well as in relation to the use of complexes of this element in catalysis,²⁵ and in photochemistry.²⁶ While Ru(III) is the predominant oxidation state under physiological conditions, Ru(II) and Ru(IV) oxidation states are readily accessible in the presence of biological reductants (*e.g.*, ascorbate or glutathione) or oxidants (O_2 or H_2O_2), respectively.^{19,27,28} Even Ru(V) has been postulated to be involved,²⁷ but the evidence for this is less certain. All three of these oxidation states (Ru(II), Ru(III) and Ru(IV)) typically form octahedral coordination compounds (mostly with relatively “soft” nitrogen and sulfur donor ligands), while typical Ru(II) organometallic complexes are tetrahedral

(piano-stool pseudo-octahedral geometry), including at least one π -bond to an arene ligand.^{19,25,26,29}

The history of development of Ru anticancer drugs has been extensively reviewed.^{19,21,28–35} Briefly, one of the earliest types of anticancer Ru complexes, proposed by Clarke and co-workers in the 1980s,^{30,36,37} were the chlorido-ammine Ru(II) and Ru(III) complexes, such as **7** in Chart 1, clearly inspired by cisplatin (**1a** in Scheme 1), and which were thought to act primarily by binding to DNA.³⁰ Among other Ru–ammine complexes tested for their cytotoxicity in cancer cell lines^{30,37} was a well-known cytological dye, ruthenium red (**8** in Chart 1), which is thought to inhibit Ca(II) transport into cells by selective binding to Ca(II)-transporting proteins.^{30,37,38}

One of the main issues with biological applications of uncharged complexes, such as **1a** or **7**, is their poor water solubility.²⁹ Therefore, the next major class of compounds to be studied (by Alessio, Sava and co-workers) were the highly water-soluble Ru(II) chlorido-dmsol complexes (where dmsol is an *S*-bound dimethylsulfoxide ligand).^{39–41} It was found that the *trans*-[RuCl₂(dmsol)₄] complex (**9** in Chart 1) was much more cytotoxic than its *cis*-counterpart.^{39,42} This is in stark contrast to the relative activities of the geometric isomers of Pt(II) complexes **1a** and **1b** (Scheme 1),⁸ which pointed to likely differences in the mechanisms of action of Ru(II) and Pt(II) complexes.⁴² Furthermore, it was established that some Ru(II) chlorido-dmsol complexes possessed anti-metastatic activity

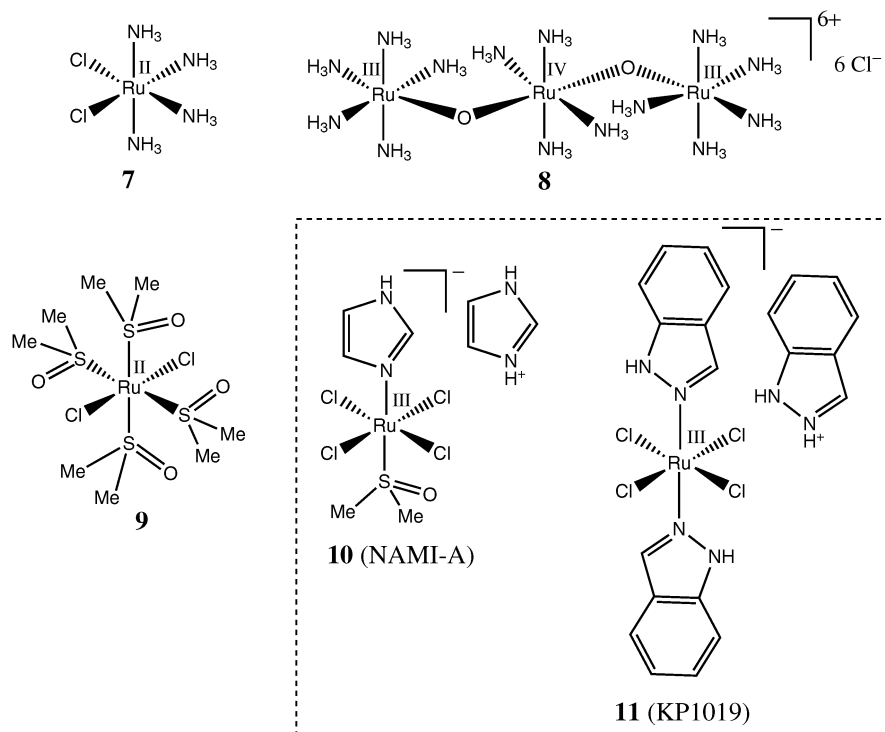


Chart 1 Representative Ru complexes with monodentate ligands tested as anticancer drugs.

(particularly in non-small cell lung cancer) while being relatively inactive against primary tumours.^{40,43} Recent developments in this area include the syntheses and biological testing of Ru(II)–dmsO complexes with chelating ligands,^{41,44} as well as of Os(II)–dmsO complexes.⁴⁵ The introduction of chelating ligands generally increases the stability of Ru(II)–dmsO complexes against aquation and hydrolysis in aqueous solutions, but no obvious correlation between the aquation rates and biological activities has been found.⁴¹

The search for more biologically active compounds related to **9** led to the development of NAMI-A (**10** in Chart 1), a specifically anti-metastatic drug that has recently completed phase I clinical trials.^{29,32,46} Since **10** undergoes aquation and hydrolysis within minutes in aqueous media at pH = 7.4 and 37 °C⁴⁷ and is practically non-cytotoxic in common cancer cell lines,⁴⁸ it is clear that its activity is not related to DNA binding in the cell nucleus.^{32,41} Interactions with actin-type proteins on the cell surface,^{49,50} or with collagens of the extracellular matrix,^{51,52} which lead to reduced mobility of invasive cancer cells, have been suggested as possible mechanisms of anti-metastatic action of **10**. Numerous analogues of NAMI-A (including Os(III) complexes) have been synthesized and characterised,^{32,45,48,53,54} but no major improvements in their anti-metastatic activity compared with the parent drug have been reported as yet.

Contemporary with the development of NAMI-A by Sava and co-workers, Keppler and co-workers discovered a Ru(III) chlorido-indazole complex, KP1019 (**11** in Chart 1). The KP1019 drug is more stable toward aquation and hydrolysis and is more readily taken up by cells than is NAMI-A,^{53,55,56} it also shows a remarkable activity against primary cisplatin-resistant colorectal tumours, but no pronounced anti-metastatic

activity.^{28,57} This drug has also recently completed phase I clinical trials.^{58,59} Since both NAMI-A (**10**) and KP1019 (**11**) complexes readily react with biological reductants (*e.g.*, ascorbate or glutathione) in protein-free model systems,^{60–62} it was suggested that the reduction of Ru(III) pro-drugs to Ru(II) species may be required for their biological activity, as in the case of Pt(IV) complexes, such as **6** in Scheme 1 (activation by reduction hypothesis).²⁸ Although experimental evidence points to an increase in anti-metastatic activity of **10** in the presence of biological reductants,⁶⁰ no direct studies of the changes of Ru oxidation states in cells and tissues (similar to those of Pt(IV) conversion to Pt(II))⁶³ have been reported as yet.

The most numerous group of cytotoxic Ru compounds are Ru(II) arene complexes, which were developed primarily by Dyson and co-workers^{19,34,64} and Sadler and co-workers,^{11,33,65} although none of these compounds has yet entered clinical trials. One of the motivations for the development of these air-stable Ru(II) complexes was the activation by reduction hypothesis, which suggested that active Ru(II) species may be formed *in vivo* from Ru(III) precursors, such as **10** or **11**.^{11,28} Typical Ru(II)–arene complexes, such as **12** (RAPTA-C)⁶⁴ and **13**⁶⁵ in Chart 2 possess Cl[−] leaving groups and aquate and hydrolyse readily at pH = 7.4 and low Cl[−] concentrations (corresponding to intracellular conditions), but the aquation equilibrium and rate are strongly inhibited by excess Cl[−] (corresponding to the blood plasma conditions), similarly to the conversion of **1a** into **2** in Scheme 1.^{66,67} The cytotoxicities of **12**, **13** and related compounds are thought to be caused by the binding of the aquation products to DNA, in a similar manner as described for **3** in Scheme 1.^{19,33} The kinetics of aquation and cellular uptake of Ru(II) arene complexes can be

fine-tuned by changing the nature of the metal centre (Ru(II) or Os(II)) or of the arene ligand, or by introducing a chelating ligand (as illustrated by the structures **12** and **13** in Chart 2);^{68–72} detailed surveys of such compounds can be found in recent reviews.^{11,19,33,34,64,65,73} As a result of the comprehensive nature of these recent reviews, it is not necessary to cover these areas in as much depth as the other areas covered in the current review, but this in no way diminishes the importance of these interesting new classes of drugs, which may emerge as being clinically significant.

Some of the recently synthesized Ru(II) arene complexes, such as **14** and **15** (Chart 2)^{74,75} are notable for their relatively high solubility in water combined with very high stability towards aquation. It is thought that **15** is taken up unchanged into the cytoplasm, where it acts as a catalyst of glutathione oxidation by O₂, which leads to an increase in cellular oxidative stress and promotes cell death *via* apoptosis.⁷⁵ Notably, the catalysis is thought to be promoted by the nucleophilic addition of the thiolato group of glutathione to the azo group of the ligand, rather than to the Ru(II) centre, in **15**.⁷⁵ This unusual mechanism may also contribute to the cytotoxicity of the [Ru^{II}(azpy)₂Cl₂] or [Ru^{II}(azpy)₃]²⁺ type complexes (where azpy are the arylazopyridine ligands similar to that in **15**), which were studied in detail by Reedijk and co-workers.^{76,77} Another recently emerged function of Ru(II)–arene complexes is the inhibition of several types of enzymes thought to be involved in cancer progression (thioredoxin reductase and catpepsin B)⁷⁸ or in the development of resistance to anticancer drugs (glutathione *S*-transferrase).⁷⁹ In both cases, a Ru complex is likely to bind to a cysteine or selenocysteine residue in the active site of an enzyme.^{78,79} Finally, some RAPTA-type complexes (analogues of **12**) have recently been shown to possess anti-metastatic activity similar to that of **10**.⁸⁰

One of the most remarkable recent findings is the high cytotoxicity of trinuclear Ru–arene clusters, such as **16** in

Chart 2 (and its analogues with various arene ligands) in ovarian cancer cell lines, while the cytotoxicity of closely related tetranuclear Ru clusters under the same conditions was at least an order of magnitude lower.⁸¹ An example of a supramolecular chemistry approach to the development of novel metal-based anti-cancer drugs involves the synthesis of cage-like polynuclear Ru(II) arene complexes that encapsulate cytotoxic Pt(II) complexes. This approach is postulated to lead to synergistic actions of both classes of complexes.⁸² Binding of Ru(III/II) complexes to antibodies, or lipid-based nanovectors, for targeted delivery to cancer cells has been proposed.^{83,84} Recently, the first example of a ¹⁰⁶Ru-radiolabelled arene complex, which can be used in the studies of metabolism of Ru anticancer drugs and in tumour imaging, has also been reported.⁸⁵ This radioisotope has also been used for a long time in the clinic in implants of metallic Ru enriched with ¹⁰⁶Ru isotope (half life ~1 year) for radiotherapy of ophthalmological cancers.⁸⁶

Representative examples of Ru(III/II) complexes with chelating ligands, tested as potential anticancer drugs, are shown in Chart 3. The chemistry of Ru(III) polyaminocarboxylato complexes (such as **17** in Chart 2) has been extensively studied,^{27,87} and they exhibit considerable cytotoxicity against common cancer cell lines.^{88,89} These complexes are characterised by unusually rapid exchange of chlorido ligands for aqua ligands or for donor groups of biomolecules. They also have a propensity for oxidation to Ru(IV) (and possibly Ru(V)) species under biologically relevant conditions (by aerial dioxygen or H₂O₂ at pH = 5–8).^{87,90} Therefore, likely modes of anticancer action of these complexes include rapid ligand-exchange reactions with active centres of enzymes (such as cysteine residues of proteases or protein tyrosine phosphatases), as well as induction of cellular oxidative stress.^{27,91,92} In addition, **17** and its analogues can affect cellular signalling by acting as NO scavengers.²⁷

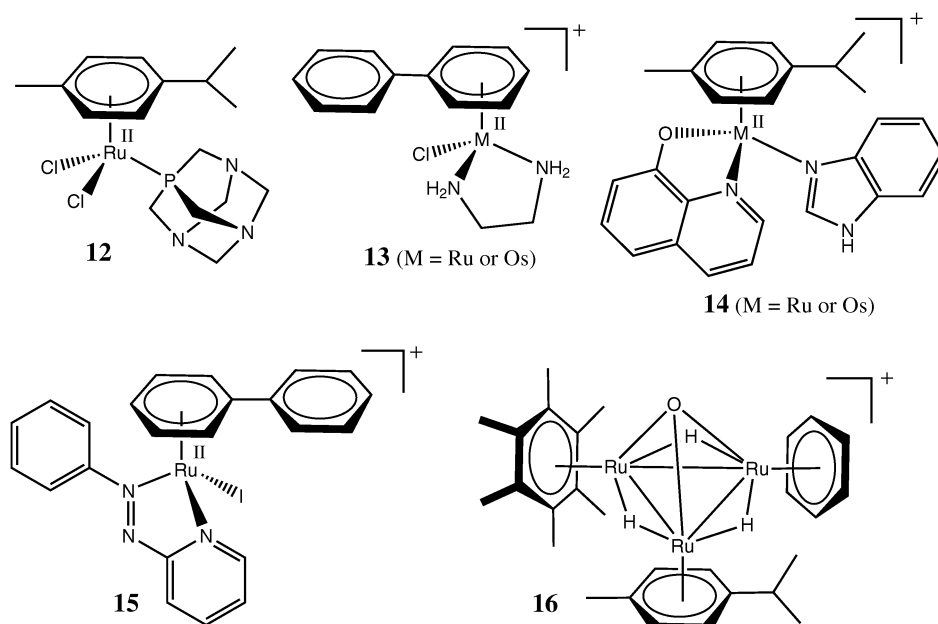


Chart 2 Representative organometallic Ru or Os complexes tested as anticancer drugs.

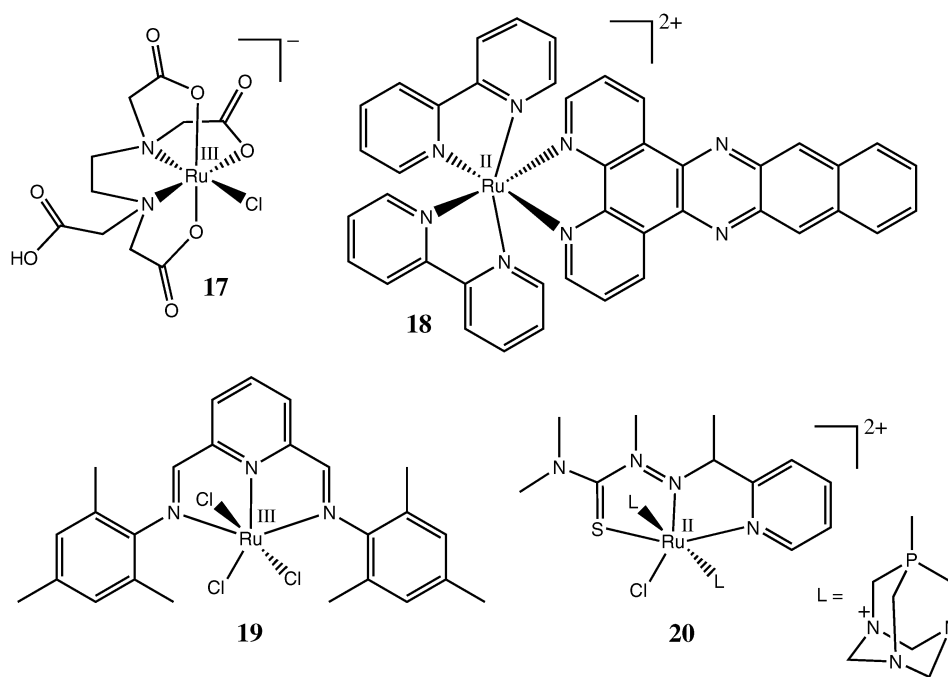


Chart 3 Representative Ru complexes with polydentate ligands tested as anticancer drugs.

Well-known Ru(II) complexes with polypyridyl ligands²⁶ have been extensively tested for their DNA-binding and cytotoxic properties, starting from the work of Dwyer and co-workers in the 1960s.^{3,30,93,94} For instance, the *in vitro* cytotoxicity of complex **18** (Chart 3) in human colon and breast cancer cell lines was comparable to that of cisplatin.⁹⁵ Unlike the complexes mentioned previously, **18** does not have any potential leaving groups, and probably acts as a DNA intercalator, due to the presence of a large aromatic polycyclic ligand.⁹³ Recently, other types of polydentate *N*-donor ligands, such as bis(arylimino)pyridine in **19** (Chart 3), have been proposed as more easily accessible and versatile alternatives to polypyridyls for the synthesis and biological screening of Ru(III/II) complexes.⁹⁶ A water-soluble Ru(II) complex with a tridentate thiosemicarbazone ligand, **20** (Chart 3), showed higher cytotoxicity towards cancer cell lines in slightly acidic media (pH = 6.0) compared with neutral media (pH = 7.4), which may be beneficial for its selective action in tumour tissues that often have somewhat higher acidity compared with the surrounding normal tissues.⁹⁷

The well-known photosensitivity of Ru(II) complexes with polypyridyl or macrocyclic ligands²⁶ prompted their testing as sensitizers for photodynamic therapy (PDT) of cancer.⁹⁸ The Ru complexes suitable for PDT have to conform to the following requirements:³⁰ (i) be both stable towards aquation and relatively non-toxic; (ii) accumulate preferentially in cancer *versus* normal cells; (iii) absorb strongly at relatively long wavelengths (640–850 nm) that are not significantly absorbed by biological tissues; and (iv) produce high quantum yields of free radical intermediates or singlet oxygen (¹O₂) on irradiation. A recent example of successful implementation of these principles is a tetranuclear Ru(II)–arene–porphyrin complex **21** (Chart 4) and its analogues, which showed

promising activity in PDT of melanoma.⁹⁹ A special case of PDT is the use of photo-induced NO donors, which lead to massive localised release of NO (a ubiquitous signalling molecule) in cancer cells and trigger their death through apoptosis.^{100,101} Recent synthesis of Ru(III)–NO complexes, such as **22** (Chart 4), which are able to release NO on irradiation with visible light (~500 nm) due to the presence of a fluorescent dye as a co-ligand, provide the necessary first step in the development of this type of Ru-based PDT agents.^{101,102}

Complex **18** (Chart 3)⁹⁵ presents an example of cytotoxicity caused by non-covalent interactions with biomolecules (in this case, DNA intercalation),⁹³ rather than by covalent binding, as shown by **3** and **4** in Scheme 1. Other examples of such interactions are provided by organometallic Ru complexes **23** and **24** (Chart 5),^{103–105} which have been designed as structural analogues of known organic anticancer drugs (tamoxifen and staurosporine, respectively, see Chart 5). These drugs (and their analogues with other metal ions, such as Fe(II))¹¹ are thought to act by non-covalent binding to specific proteins involved in the development of cancer, such as oestrogen receptors (for tamoxifen)¹⁰³ or cyclin-dependent kinases (for staurosporine).¹⁰⁴ However, whereas Fe-tamoxifen (ferrocifen) is cytotoxic against both ER(+) and ER(–) breast cancer cells, the structurally similar ruthenocene adduct of ferrocifen (**23**) has antiproliferative action against ER(+), but not against ER(–) breast cancer cells,¹⁰⁶ although it is unclear which is more likely to be more active *in vivo*. In particular, metal complexes like **24** provide a very attractive synthetic alternative to polycyclic systems, such as staurosporine, which are very common among natural biologically active compounds.⁴ Even more attractive is the use of supramolecular chemistry techniques for self-assembly of elaborate three-dimensional structures, such as a triple-helicate

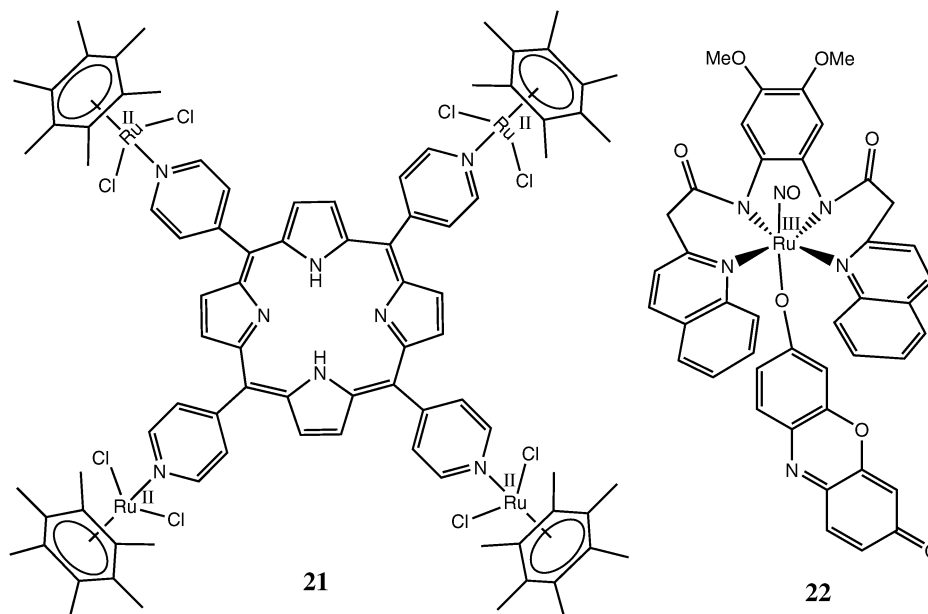


Chart 4 Representative Ru complexes tested as active agents for photodynamic therapy.

Ru(II) complex **25** (Chart 5).¹⁰⁷ The size and shape of complex **25** resembles that of zinc finger proteins, and it shows considerable cytotoxicity in cancer cell lines (much higher than that of a related dinuclear Ru(II) complex with non-helical structure),¹⁰⁸ presumably due to the tight non-covalent binding to DNA. Generally, the use of metal (including Ru) ions as scaffolds for the assembly of rigid three-dimensional structures designed for non-covalent interactions with specific biological targets is likely to develop into one of the leading directions in medicinal chemistry.^{4,21,109}

Interactions with biomolecules and general pathways for biological activation of ruthenium anticancer pro-drugs

Numerous reviews on Ru anticancer drugs, published in the last five years, have tended to concentrate either on a single compound, such as NAMI-A (**10**)^{29,31,32} or KP1019 (**11**),^{28,58,110} or on a group of complexes with similar ligands, such as ammine,³⁰ polypyridyls,^{30,93,94} polyaminecarboxylates,²⁷ or arenes,^{19,33,34,65,73} and their different modes of reactivity in biological media. On the other hand, very recent evidence in the literature points to similarities in the mechanisms of action of various types of Ru anticancer complexes. For instance, specific anti-metastatic activity, which was until recently regarded as a unique property of NAMI-A and its close analogues, has also been demonstrated for RAPTA-type Ru(II)-arene complexes.⁸⁰ As with NAMI-A, the anti-metastatic activity of Ru(II)-arene complexes appears to be related to interactions with the extracellular matrix and the cell surface, rather than with DNA in the cell nucleus.⁸⁰ Another example is a common mechanism of enzyme inhibition, based on binding of the Ru ion to *S*- or *Se*-donor groups in the active sites of enzymes that has been proposed for both Ru(III)-polyaminecarboxylato^{91,92} and Ru(II)-arene^{78,79} complexes.

In this section, we bring together what is known about the chemistry and biochemistry of the diverse range of Ru drugs to hypothesise whether all of the activities of the Ru drugs can be described within the constraints of common chemical pathways for the reactions in biological fluids, extracellular matrices, cell surfaces, and intracellular targets. This hypothesis attributes the different biological activities of the Ru complexes to the relative rates by which the Ru complexes react with various biomolecules within or outside the cells, or on the cell surfaces (Scheme 2). At this stage, there are considerable gaps in knowledge about the reactivities of the drugs along these pathways. As such, this review has been designed, in part, to stimulate new research to fill these gaps.

From the data presented in the previous sections, it is clear that interactions with biological macromolecules are crucial for both activities and deactivation of Ru anticancer drugs. In the past, most of the studies have concentrated on the binding of Ru complexes to DNA and its components,^{30,37,93,111,112} while more recently, the main focus has shifted to their interactions with blood plasma proteins.^{58,113} Scheme 2 outlines two main metabolic routes of Ru complexes (designated as L_nRu-X in Scheme 2, where L are the strongly bound ligands such as arenes or *N*-donors, and X are the leaving groups such as chlorido or carboxylato ligands): cell permeability (either by passive diffusion or by specific transport mechanisms) from extracellular to intracellular compartments, and their aquation and binding to biomolecules specific to each compartment.

Intravenously administered Ru complexes can undergo aquation to various extents in blood plasma, followed by their binding to serum proteins, such as albumin or transferrin (Scheme 2).^{58,113} While the aquation and protein binding reactions may be catalysed by biological reductants, such as ascorbate (through the formation of kinetically labile Ru(II)-Cl intermediates), recent kinetic studies (performed mostly with **11**) suggested that reduction to Ru(II) is unlikely to play a major role in the efficient binding of Ru(III)

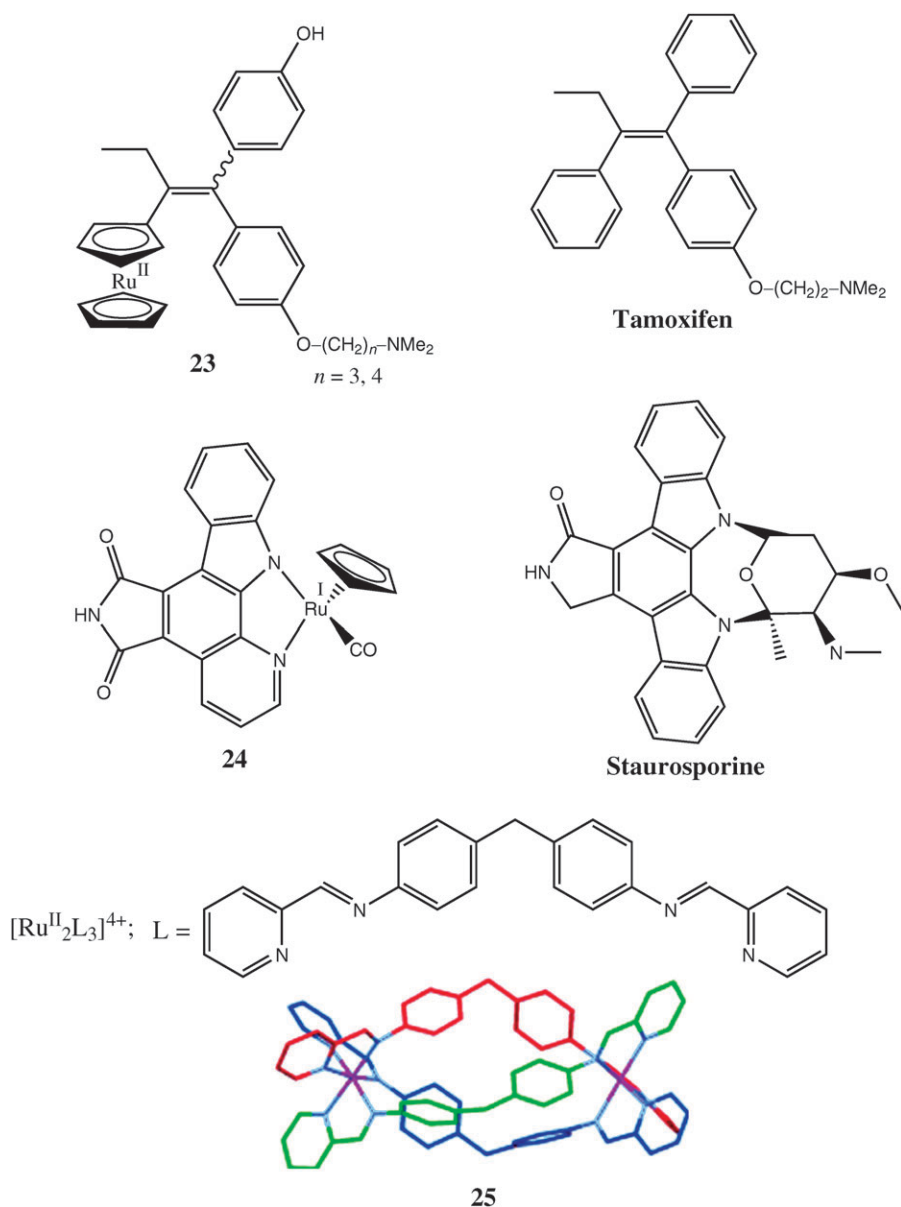
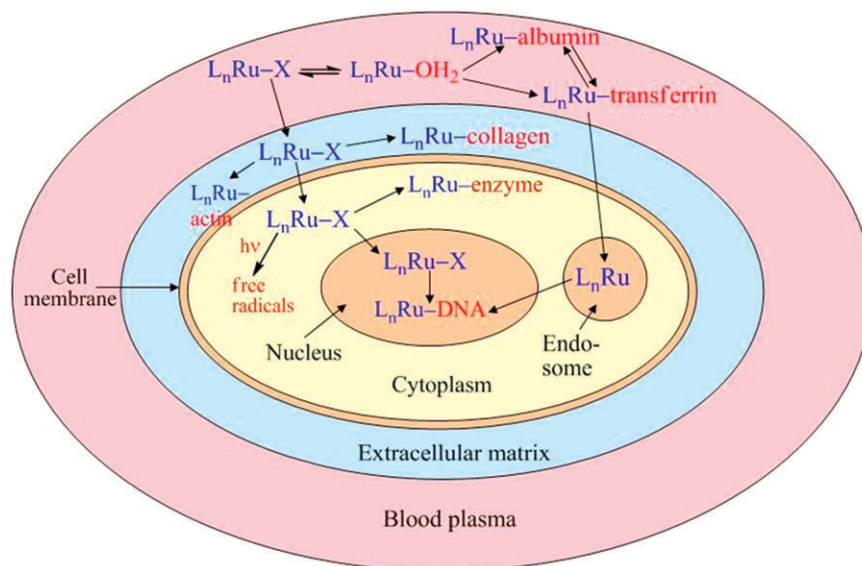


Chart 5 Representative Ru complexes designed as structural analogues of known biologically active compounds.

anticancer drugs to blood proteins.^{58,114} In the case of **11**, at least 90% of Ru was bound to albumin (by far the most abundant protein) in the bloodstream, but an equilibrium is thought to exist between Ru species bound to albumin and transferrin (Tf, the main Fe(III) transport protein), so that the former acts as a depot of Ru ions, and the latter might provide their active transport to the cells.^{28,58}

To our knowledge, **11** is the only anticancer Ru complex for which Tf binding has been studied in detail.^{58,113} Strong binding of Ru(III) from **11** to histidine residues of Tf has been suggested based on the results of circular dichroism (CD) spectroscopy, electrospray mass spectrometry (ESMS) and gel filtration studies,¹¹³ as well as on X-ray diffraction data for **11**-lactoferrin adducts (lactoferrin is a protein closely related to Tf),¹¹⁵ although it is unclear to what extent Ru(III) binds specifically to the Fe(III) binding sites of Tf. Cellular uptake studies of **11** in the presence of Tf suggested that the

highest uptake is achieved when one of the two binding sites of Tf is loaded with Ru(III), and the other one with Fe(III).⁵⁸ It was proposed that Ru(II) is released from the Ru(III)-Tf complex inside the cells, following its reduction by ascorbate or glutathione.^{28,58} However, if Ru(III) is indeed taken into cells in a stable complex with Tf, it would follow the metabolic pathway for a Fe(III)-Tf complex, including binding to a Tf receptor on the cell surface, then encapsulation within an endosome (Scheme 2), from which it might be released as a Ru(II) complex (with unspecified biological ligands) by a combination of enzymatic reduction and decrease of pH within an endosome.¹¹⁶ However, given the inert nature of Ru(II) imidazole complexes,¹¹⁷⁻¹¹⁹ it is unclear whether the postulate that Ru(II) would be released from transferrin under these conditions can be justified. An alternative mechanism of the anticancer activities of Ru(III/II) complexes may involve interference with Fe uptake and metabolism.¹¹⁶ Another metal



Scheme 2 Proposed generalised pathways of action of Ru anticancer drugs (L are the tightly bound ligands and X is the leaving group).

ion that is thought to owe its anticancer activity to the resemblance of its uptake mechanism to that of Fe(III), namely Ga(III), is known to bind specifically to Tf and to be transported actively into cells. Within the cells, further metabolism of Ga(III) is thought to be blocked due to the inability of Ga(III) to be reduced within endosome, so that Ga(III) is likely to act by starving cancer cells of vital Fe(III).¹²⁰

Although it has not been discussed in the literature, histidine-bound Ru(III/II) sites in Tf complexes may undergo acid-catalysed *N/C* linkage isomerisation reactions similar to those observed with a range of imidazole-containing ligands^{118,119} that would most likely occur in the acidic and reducing environment of endosomes (pH ~ 5),¹¹⁶ or hypoxic tumours (pH ~ 6).²⁰ This possibility requires further investigation, because of the potential biological significance of such *C*-bound imidazole linkage isomers in Ru(III/II)-Tf complexes, and imidazole-containing anti-cancer drugs, as a result of the strong labilising effects of *C*-bound imidazoles on both *cis* and *trans* auxiliary ligands. Thus, the presence of even small equilibrium concentrations of such linkage isomers would lead to greatly enhanced reactivity compared with Ru adducts containing only *N*-bound imidazoles.¹²¹ At present, nothing is known about the ability of Ru(III) to follow the Fe(III/II) metabolic pathway after its uptake by cells,⁵⁸ and more extensive studies of the interactions of Tf with various types of Ru complexes will be required before definitive conclusions can be reached. Another potentially important aspect of reactivity that has not been adequately discussed in the literature and warrants closer scrutiny for the dmsO-containing pro-drugs, is *S/O*-dmsO linkage isomerisation reactions, which are known to be oxidation-state dependent.¹²² Such linkage isomerisation reactions have a marked effect on the redox behaviour and kinetics and thermodynamics of ligand-exchange reactions of imidazole and dmsO complexes, and there is a gap in knowledge about the potential importance of these processes in the diverse biological environments encountered by the drugs.

If a Ru complex escapes protein binding in the blood plasma and subsequent excretion, it can diffuse into the extracellular matrix (Scheme 2), and form adducts there with collagens or cell surface proteins (such as actins), which may be responsible for the anti-metastatic action of **10**^{49,50,52} and Ru(II)-arene complexes.⁸⁰ Any unreacted Ru complex can further diffuse through the cell membrane into the cytoplasm (Scheme 2), and bind to the active centres of various enzymes, either covalently^{78,79,92} or non-covalently.^{4,21} Alternatively, Ru complexes can trigger intracellular oxidation reactions, either through activation by visible light (photodynamic therapy, Scheme 2)⁹⁹ or through depletion of cellular reductants.⁷⁵ Finally, Ru species that reach the cell nucleus either by diffusion or by active uptake involving the transferrin pathway (Scheme 2), can form DNA adducts, either by covalent binding,¹¹² or non-covalently (by intercalation or by mimicking zinc finger proteins).^{93,107} The multitude of extracellular and intracellular targets available for interactions with Ru complexes, as illustrated in Scheme 2, may provide a key to the ability of these complexes to overcome tumour resistance,^{123,124} since cancer cells are less likely to develop a protection mechanism against drugs exerting multiple cytotoxic routes compared with those targeting a particular biochemical pathway.¹²⁵

According to Scheme 2, the predominant mode(s) of biological action for each Ru complex is(are) likely to be determined by the relative rates of its cellular uptake *vs.* extracellular aquation, hydrolysis and protein binding. For instance, extracellular interactions are likely to be responsible for the anti-metastatic activity (and lack of significant cytotoxicity) of hydrophilic and labile Ru complexes such as **9** or **10**,^{41,126} while **11**, which is more resistant to aquation and is more readily taken into cells, acts primarily as a cytotoxic drug.⁵⁵ Arene complexes of Ru(II) (related to **12**), previously evaluated mainly for their cytotoxicity,^{71,72} have been recently shown to possess anti-metastatic properties similar to those of **10**.⁸⁰ Further studies are required to determine whether the cytotoxic *vs.* anti-metastatic properties of Ru(II) arene

complexes correlate with their aquation and cellular uptake rates. As shown in Scheme 2, the ability to follow the path of a Ru complex from blood plasma to the cell and its compartments, as well as the changes in the chemical state of Ru along this pathway, is crucial for an understanding of the mechanisms of anticancer activity of Ru complexes.

To date, extensive research on the interactions of Ru anticancer drugs (primarily **11**) with blood serum proteins^{58,113} has relied mainly on capillary electrophoresis^{127–129} or size-exclusion chromatography¹³⁰ coupled to ICPMS (inductively coupled plasma mass spectrometry, used for determination of Ru). While providing quantitative information about the extent of Ru binding to various types of proteins, these techniques do not detect changes in the oxidation state or coordination environment of Ru (e.g., cleavage of ligands from the original complex). In recent years, a synchrotron-based technique, X-ray absorption spectroscopy (including X-ray near-edge structure or XANES and X-ray absorption fine structure or XAFS components) has developed into a powerful tool for studies of metal speciation in biological systems,^{92,131–135} such as cellular metabolism of Pt anticancer drugs^{63,136,137} or binding of **10** to bovine serum albumin.¹³⁸ In the latter case, a combination of L₃- and K-edge spectra of Ru and K-edge spectra of Cl and S was used to cross-reference the changes that occur in the metal centre and in the ligands of **10** upon binding to a protein molecule.¹³⁸ It was concluded that the metal remains in the Ru(III) oxidation state and the main reaction is Cl[−] ligand substitution (which may also be the result of aquation of **10** prior to the protein binding, see Scheme 2).¹³⁸ However, this conclusion is not necessarily valid, since redox catalysis of Ru(III) substitution reactions can occur with amounts of Ru(II) that are too small to be measured by the XANES technique. Such Ru(II) catalysis of substitution of Ru(III) is well-known,¹³⁹ and chlorido–Ru(II) complexes are very labile to the extent that they could catalyse the substitution reactions with biomolecules *via* aqua intermediates before reoxidation of the biomolecule Ru(II)-adducts to Ru(III).¹⁴⁰

Our group has previously used XANES spectroscopy for detailed studies of metabolism of chromium compounds (including carcinogenic Cr(VI) and purportedly anti-diabetic Cr(III) nutritional supplements) in biological fluids and in cultured cells.^{141,142} The main advantages of XANES spectroscopy for such studies are the following: (i) specificity toward the studied metal ion, regardless of the physical state and chemical composition of the sample; and (ii) high sensitivity to small changes in the coordination environment of metal ions.¹⁴³ Multiple linear regression analyses of XANES spectra with the use of a library of model Cr complexes led to assignment of the chemical natures of Cr metabolism products.^{141,142} Crucially, a comparison of XANES spectra of Cr(III) metabolism products in whole cells and in subcellular fractions pointed to a re-distribution and changes in the coordination environment of Cr(III) caused by the cell lysis and separation procedures.¹⁴¹ These changes mean that metal–protein complexes isolated by chromatographic techniques do not necessarily correspond to the species formed in metal-treated cells, and the use of XANES spectroscopy provides a unique opportunity to delve into changes that take place.^{141,143}

Recently, we applied the same principles to the studies of transformations of typical Ru(III) anticancer drugs, **10** and **11**, in cultured mammalian cells, as well as in cell culture media and in blood serum (Z.-J. Lim, Y. Y. Gwee, A. Levina, A. Mitra and P. A. Lay, to be submitted). Unlike our previous studies on Cr^{141,142} and the published data on Ru,¹³⁸ we found that the consideration of whole spectra, including XANES and XAFS regions, is essential for the analysis of changes in the coordination environment of Ru compounds in biological media (Fig. 2). For instance, the loss of Cl[−] ligands during the decomposition of **11** in cell culture medium is manifested in a decrease in the number and intensity of post-edge maxima in the spectra, while changes in the coordination environment of model Ru(III) complexes from *N*- to *O*- donor groups cause significant shifts in the positions of these maxima (at 22 200–22 400 eV, Fig. 2). Multiple linear regression analysis indicated that Ru(III) is bound mainly to *N*-donors and to a lesser extent to *O*- and *Cl*-donors in the decomposition products of **11** (Fig. 2), while gel filtration studies confirmed that the resultant Ru(III) species were fully protein-bound (the protein source was fetal calf serum added to the medium). We expect that further studies in this direction will shed new light onto the fate of Ru anticancer drugs in biological systems.

An even more promising synchrotron-based technique for the study of biotransformations of Ru anticancer drugs is X-ray fluorescence microprobe imaging,¹⁴⁴ including studies of Ru distribution in single mammalian cells or in cancer tissues (used previously for Pt anticancer drugs)^{63,136,145} or in gels after the separation of lysates from metal-treated cells.^{132,144,146} A combination of microprobe X-ray fluorescence imaging and XANES spectroscopy allows for the determination of the oxidation states and coordination environments of metal ions within single cells and their organelles,^{92,136,147,148} which is likely to provide crucial information for understanding the mechanisms of action of Ru anticancer drugs, including clarification of the role of transferrin in Ru(III) transport⁵⁸ and testing of the activation by reduction hypothesis.²⁸ These can be combined with correlative microscopic studies, such as those involving vibrational microprobe images, to examine how the drugs affect the biochemistry of the cells.^{149,150}

Conclusions

For two decades, there was little overlap between the publications of several research groups that have developed different types of Ru anticancer drugs, including NAMI-A,³² KP1019⁵⁸ and Ru(II)–arene complexes.^{33,34} However, recent trends point to the likelihood of common pathways of biological activation for all types of Ru complexes (Scheme 2) that include competing processes of extracellular protein binding and cellular uptake. Similar to Pt anticancer drugs, the latter process is likely to be responsible for the cytotoxicity of Ru complexes in primary tumours (e.g., for KP1019).⁵⁸ Unlike Pt complexes, where binding to extracellular proteins is thought to cause the loss of biological activity,¹⁰ binding of Ru complexes to extracellular matrix proteins or to the cell surface is likely to be responsible for the anti-metastatic properties of NAMI-A and of some

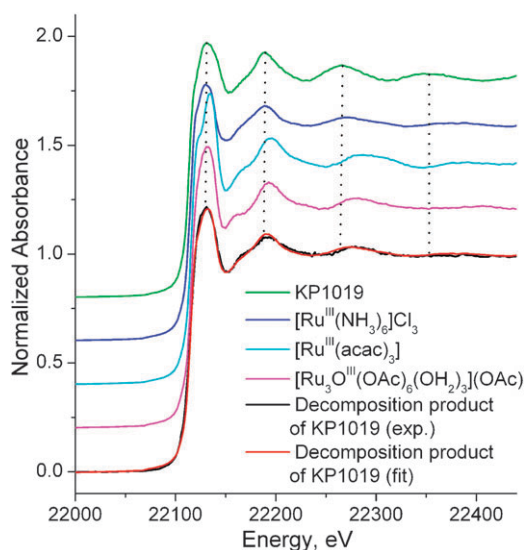


Fig. 2 Representative K-edge X-ray absorption spectra of model Ru compounds (solids mixed with boron nitride in ~1 : 10 ratio) and decomposition products of KP1019 in cell culture medium (Advanced DMEM from Invitrogen, supplemented with 2% fetal calf serum and 0.50 mM KP1019, incubated for 4 h at 37 °C, then freeze-dried). The spectra were collected at the Australian National Beamline Facility (beamline 20B at the Photon Factory, Tsukuba, Japan) at 14 K, using the fluorescence detection mode (36-pixel Ge detector). The experimental spectrum of the decomposition products of KP1019 (black line) was fitted (red line, $R = 0.9994$) with a linear combination of spectra of model compounds: $[\text{Ru}^{\text{III}}(\text{NH}_3)_6]\text{Cl}_3$, $60 \pm 2\%$; $[\text{Ru}^{\text{III}}(\text{acac})_3]$, $14 \pm 2\%$ (acac = acetylacetonato = 2,4-pentanedionato(-)); $[\text{Ru}_3^{\text{III}}\text{O}(\text{OAc})_6(\text{OH})_3](\text{OAc})$, $16 \pm 3\%$; and KP1019 (**11** in Chart 1), $10 \pm 2\%$. Details of experiments and data processing were similar to those described previously (ref. 141 and 142).

Ru(II)–arene complexes.^{32,80} The main factors that determine the mode of action of a Ru complex appear to be its lipophilicity (favouring cellular uptake) and the presence of labile ligands such as chlorido or carboxylato (ligands that favour extracellular binding). The possibility of transferrin-mediated uptake of some Ru(III) complexes into cells, followed by intracellular reduction to produce reactive Ru(II) species, has been suggested,²⁸ but is not yet firmly established and is doubtful given the inert nature of Ru(II)–imidazole bonds.^{118,121} A better understanding of chemical transformations of Ru complexes in biological media is required for the design of compounds with predictable properties, particularly of new types of anti-metastatic drugs. The use of synchrotron-based techniques, such as X-ray absorption spectroscopy and microprobe X-ray fluorescence mapping, is likely to be crucial for this purpose.

Acknowledgements

The research has been supported by the Australian Research Council Discovery grants (DP0208409, DP0664706 and DP0984722) to P. A. L., including ARC Professorial Fellowships. X-Ray absorption spectroscopy (XAS) was performed at the Australian National Beamline Facility (Photon Factory, Tsukuba, Japan) with support from the Australian Synchrotron Research Program, which is funded

by the Commonwealth of Australia under the Major National Research Facilities program. We thank Prof. M. Nomura (Photon Factory, Tsukuba, Japan), Drs G. J. Foran and J. Hester (ANSTO, Australia), and Dr J. B. Aitken (University of Sydney, Australia) for their help with XAS experiments and Prof. T. W. Hambley for his helpful comments on the manuscript.

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