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Systematic Design of a Targeted Organometallic Antitumour Drug in Pre-clinical Development

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Abstract: Organometallic ruthenium compounds that are effective anticancer and antimetastasis agents are currently under intensive investigation (see also the article by Peter Sadler in this issue). This article provides a personal account of the way in which we have exploited the different properties of the organoruthenium (and osmium and rhodium) compounds to rival traditional DNA-binding platinum drugs, culminating in the discovery of a non-classical molecule that is in an advanced stage of pre-clinical evaluation.

Keywords Anticancer drugs · Antimetastasis drugs · Bioorganometallic · Drug resistance · Glutathione-S-transferase · Ruthenium · Targeted chemotherapy

Introduction

The medicinal properties of inorganic (coordination) compounds has been extensively studied due to their considerable success in the clinic, notably cisplatin and other platinum compounds,[1] with ruthenium compounds recently commencing clinical trials.[2] Organometallic compounds have also had clinical applications for a long time, a prominent example being the main-group anti-syphilis drug Salvarsan® introduced into the clinic in 1910,[3] although its structure was only recently elucidated.[4] Transition metal-based metallocenes were evaluated as anticancer compounds shortly after the discovery of cisplatin,[5] in particular, titanocene dichloride has been extensively studied and reached phase II clinical trials, which were only recently discontinued principally due to formulation problems.[6] Nevertheless, the last decade has witnessed many promising developments in the field of medicinal chemistry, with organometallic compounds displaying anticancer and antimetastasis activity, and finding applications as antibiotics, antiviral and antiparasitic agents.[7]

The unique properties of organometallic compounds that makes them so promising in a range of medicinal applications are not easily quantified, but it appears that the increased stability that many exhibit over conventional coordination compounds is advantageous. The apparent advantage organometallic compounds may offer over organic molecules is also important, with increased drug uptake being one key factor, and the potential for comparatively facile design of multi-functional compounds (see below). Thus, it appears that organometallic compounds integrate the best features of inorganic and organic compounds, although much remains to be learnt in this respect.

Why Ruthenium–Arene Complexes?

Our initial reasons for wishing to study the anti-proliferatine properties of ruthenium(II)–arene complexes were based on the notion that, to some extent, they mirror square-planer platinum compounds like cisplatin. In the simplest sense, two chloride ligands which are able to undergo hydrolysis are present, as in cisplatin, even involving a similar substitution mechanism by virtue of an 'arene-slippage' process. Our initial studies on the anticancer properties of ruthenium(II)-arene compounds[8] began in the mid-1990s – undertaken while I was at Imperial College, and at the same time developing an interest in biphasic synthesis and catalysis. In particular, we were using/developing methods to transfer organometallic catalysts between various biphasic systems including water, organic solvents and ionic liquids. This led us to predict that the phosphorus donor ligand pta that has been extensively studied by Darensburg and others (including my own group) in a catalytic context,[9] would provide hydrophilic properties that would be nicely counterbalanced by the hydrophobic arene region in a series of compounds based on ruthenium (see below).

The other attractive feature of the ruthenium–arene unit, that was subsequently developed after I moved to the EPFL, is that it represents an excellent scaffold on which to build functional organic segments for targeted chemotherapy (Fig. 1). Ferrocene compounds have been used in this way for quite some time. Notably, the pioneering studies of Jaouen and his group who have modified selective estrogen receptor modulator ligands with various organometallic fragments including the ferrocenyl group.[10]

In addition, ruthenium is an attractive alternative to platinum: besides the rich synthetic chemistry, ruthenium has a range of oxidation states, Ru(II), Ru(III) and Ru(IV), accessible under physiological conditions,[11] and ruthenium compounds tend to be generally less toxic than platinum compounds. In terms of mode of action, the ability of ruthenium to mimic iron

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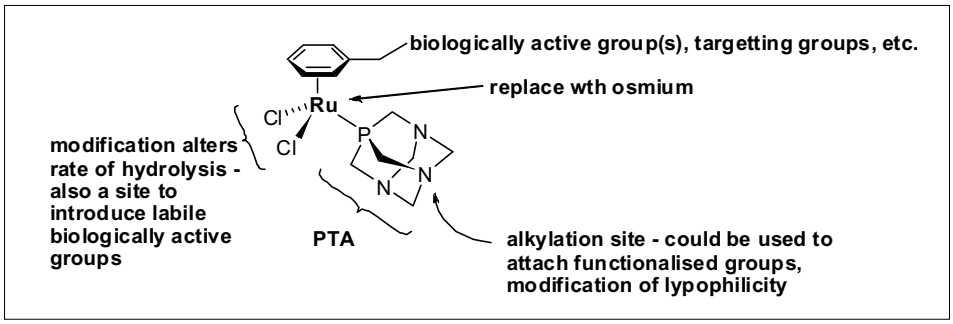

Fig. 1. Generic RAPTA structure Ru(η^6-arene)Cl_2(pta) with potential sites for modification indicated. Apart from ruthenium compounds we pioneered osmium analogues which also show promise.

in the binding to biological molecules, such as albumin and transferrin, and the possibility of proteomic targets is interesting,[12] since anticancer drugs are usually used in combination therapy and excellent DNA damaging drugs are already available, with alternative targets identified in recent years by molecular oncologists.[13]

The low toxicity of indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)], KP1019, is attributed, at least in part, to transferrin-mediated drug transport,[14] with KP1019 binding strongly to transferrin in the iron-binding pockets.[15] Indeed, its imidazolium analogue, which binds more weakly to transferrin, is taken up less effectively by transferrin and is less cytotoxic *in vitro*.[16] Another possible mechanism in play is that the drug is activated by reduction, from Ru(III) to Ru(II), selectively in hypoxic tumour tissue by endogenous bioreductants such as gluthathione.[17] It appears that KP1019 induces apoptosis in colorectal cell lines predominantly by the intrinsic mitochondria pathway and that DNA could be a target, although studies have shown that the DNA lesions formed by KP1019 are different to those of cisplatin.[18] Imidazolium *trans*-[tetrachloro (DMSO)(imidazole)ruthenate(III)], NAMI-A, binds within minutes to plasma proteins,[19] inhibits lung metastases formation and reduces metastases weight without affecting the primary tumour.[20] Indeed, both *in vitro* and *in vivo* data appear to exclude DNA as the primary target, in line with the observation that the binding of NAMI-A to DNA is much weaker than platinum complexes.[21] KP1019 and NAMI-A are both progressing through clinical trials.[2]

Activity

The prototype ruthenium–arene compound emanating from our laboratory is [Ru(η^6-cymene)Cl_2(pta)], termed RAPTA-C (Fig. 2).[22]

RAPTA-C has been the subject of detailed reactivity, computational, *in vitro* and *in vivo* studies. In aqueous solution, the chloride ligands may be hydrolysed, the extent of which is dependant upon the concentration of the complex in solution, the amount of chloride present and the pH.[23] Typically, however, hydrolysis is suppressed in the blood plasma where chloride concentration is *ca.* 100 mM, but occurs once the compound penetrates into the cell cytoplasm where chloride concentrations are much lower, and represents a possible activation pathway for the compound. However, RAPTA-C derivatives in which the chlorides have been replaced by chelating carboxylate ligands (Fig. 2) that resist hydrolysis display very similar *in vitro* activity to RAPTA-C.[24] It is not unreasonable to assume that arene slippage ($\eta^6 \leftrightarrow \eta^4$) may therefore be important in any activation pathway; such a mechanism has been characterized in catalytic processes.[25] An arene slippage process in the context of drug activation is still under investigation in our laboratory. Additionally, the N-site on the PTA ligand may be protonated, and in RAPTA-C or the hydrolysed products the pKa for this process is quite low, *ca.* 2–3, and is unlikely to occur in a physiological environment. In general, reaction of RAPTA-C with model oligonucleotides or proteins results in adducts in which both chlorides are lost (as expected following hydrolysis), but also, in some cases loss of the arene rings is observed.[26] Thus, the arene appears to be more labile than the PTA ligand and this is substantiated by calculations. It is difficult to imagine how an adduct which has lost both chlorides and the arene could bind to DNA, since five coordination sites must be satisfied, which would suggest that DNA is an unlikely target, and *in vitro* studies have shown that proteins and RNAs are more probable targets.[27] Such data is in accordance with NAMI-A, and the similarity between RAPTA-C and NAMI-A does not end here. *In vitro*, RAPTA-C and NAMI-A exhibit a very low activity, yet *in vivo*, both compounds inhibit lung metastases in CBA mice bearing the MCa mammary carcinoma, reducing their weight and number, although only mild effects on primary tumours are observed.[28] They also exhibit low general toxicity combined with excellent clearance rates. In fact, RAPTA-C resulted in 40% of animals being completely free of metastasis after treatment and in general only drug combination therapies showed better cure rates.[29]

Modulating Activity

Modification of the basic RAPTA framework has been undertaken with a view to understand its mode of action and to improve its efficacy. In an attempt to increase the cytotoxicity of the RAPTA compounds functional groups with potential hydrogen bonding substituents were tethered to the arene,[30] since this approach has been shown to increase their affinity of metal-based drugs to DNA/RNA.[31] However, this modification actually resulted in a decrease in toxicity towards cancer cells (traced to decreased uptake) and an increase in toxicity to non-tumourigenic cells. Experiments

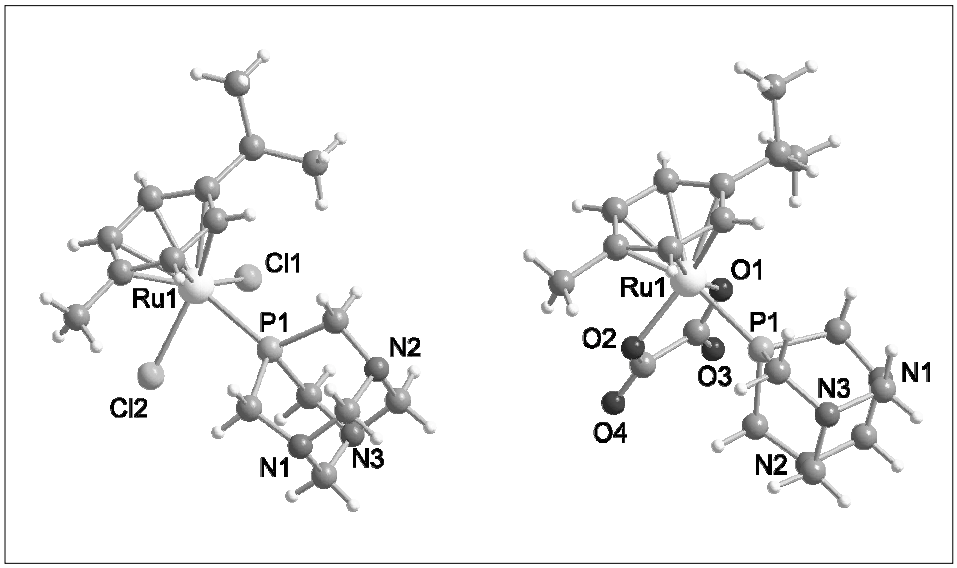

Fig. 2. Solid-state structure of (left) RAPTA-C and (right) OxaloRAPTA-C

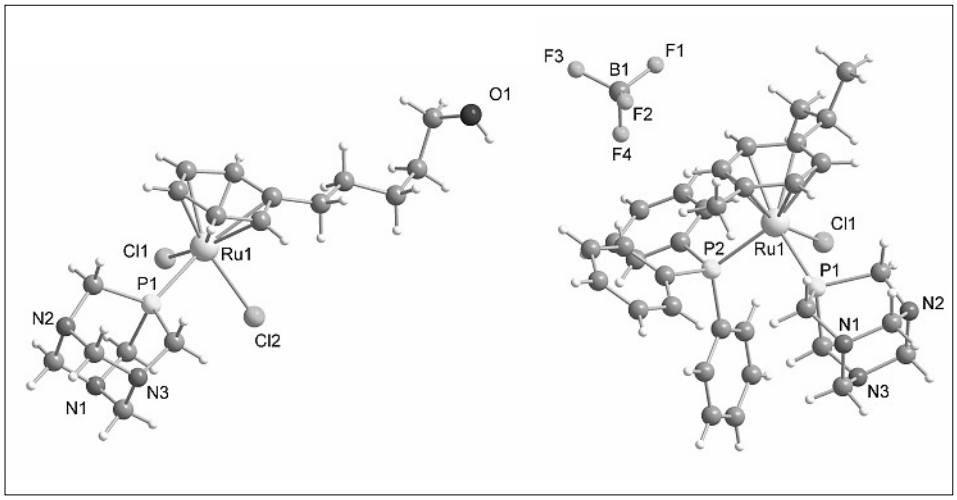

Fig. 3. Solid-state structure of (left) [Ru(η^6-$C_6H_5(CH_2)_5OH$)Cl_2(pta)] and (right) [Ru(η^6-cymene)Cl(PPh_3)(pta)]BF_4; examples of compounds which modulate the hydrophilicity/hydrophobicity of the RAPTA structure and significantly modify the type of interactions with various biomolecular targets

showed, however, that binding with a model oligonucleotide increased. Using the same functionalised arenes, but incorporating a bulky hydrophobic ligand into the RAPTA structure, resulted in increased drug uptake and increased toxicity, but selectivity also decreased relative to RAPTA-C (Fig. 3). In fact, from a series of experiments it would appear that as the capacity for DNA binding increases, at the expense of selective protein interactions, the general toxicity of the RAPTA drugs increases, which would potentially lead to more side-effects in patients. Thus, modifying the RAPTA framework to have increased interactions with proteins, ideally specific interactions, should result in a superior drug (see below).

Various other comparatively simple modifications have been made, but none resulted in a major improvement in activity. For example, replacing the arene for other isoelectronic ligands is possible without causing major changes to activity/selectivity *in vitro*.[32] As mentioned above, replacement of the chloride ligands for hydrolysis-resistant chelates (or other simple groups) did not have any significant effect on *in vitro* activity although very hydrophobic groups were recently found to significantly increase uptake and cytotoxicity.[33] Use of Me-PTA in place of PTA decreases selectivity, presumably due to increased DNA interactions due to the positive charge. Perhaps most remarkably, changing the metal itself,[34] *e.g.* for rhodium or osmium (Fig. 4) does not influence cytotoxicity to any great extent on the cell lines tested.

Targeted Approaches

Of all the different RAPTA compounds developed (see above) none proved to be significantly more effective than RAPTA-C. From the *in vivo* study, which was very positive in terms of activity (including also primery tumours in a recent study) and pharmacokinetic parameters, the main limitation of the RAPTA compounds is that high doses are required for effective treatment, although the lethal dose is very low for a metal drug, *i.e.* comparable to paracetamol. While the various modifications described in the previous section led to increased cytotoxicity, the general toxicity also increased in an unfavourable ratio or, for example, addition of functional groups such as benzocrown and imidazolium moieties improved cytotoxicity it was not to a significant degree to warrant further studies. Thus, we decided to adapt a method we had developed for the rational design of platinum drugs for a more targeted chemotherapeutic approach[35] to the RAPTA system. As a strategy to enhance drug efficacy, RAPTA complexes designed to inhibit Glutathione-S-Transferases (GST), a cytosolic detoxification enzyme associated with drug resistance, were developed (Fig. 5),[36] since inhibition of GST should allow the RAPTA unit to operate at lower doses. RAPTA complexes conjugated *via* the arene ring to ethacrynic acid, a known inhibitor of GST enzymes, were found to be effective GST inhibitors with significantly increased cytotoxic activity, *i.e.* showing comparable or superior cytotoxicity to cisplatin in various cell lines known to contain elevated levels of GST. Inhibition assays, mass spectrometry binding experiments and X-ray structures of the ruthenium complexes bound to the protein support our hypothesis that these compounds act in a bifunctional manner, *i.e.* inhibiting the enzyme and inducing cytotoxicity following cleavage of the inhibitor from the ruthenium. Higher cytotoxicities are observed for the ethacrynic acid-RAPTA conjugate ethaRAPTA-N1 (Fig. 5) than cisplatin in certain cisplatin resistance cell lines characterised by high levels of GST.

Other enzyme targets have also been investigated, for example Pgp, one of the key proteins involved in multidrug resistance.[37] In our initial studies we attached modified known phenoxazine-type Pgp inhibitors to the ruthenium(II)–arene centre *via* imidazole linkages (Fig. 6). However, an anthracene structural analogue that was found to be inactive on its own was far more effective than the known inhibitors once coordinated to the ruthenium(II) centre. Studies showed that the complex induces cell death *via* inhibition of DNA synthesis. Moreover, since the complex is fluorescent, its uptake in cells was studied, and relative to the free anthracene-based ligand, uptake of the complex is accelerated and accumulation of the complex in the cell nucleus was observed. While we had not anticipated these additional benefits the accumulation

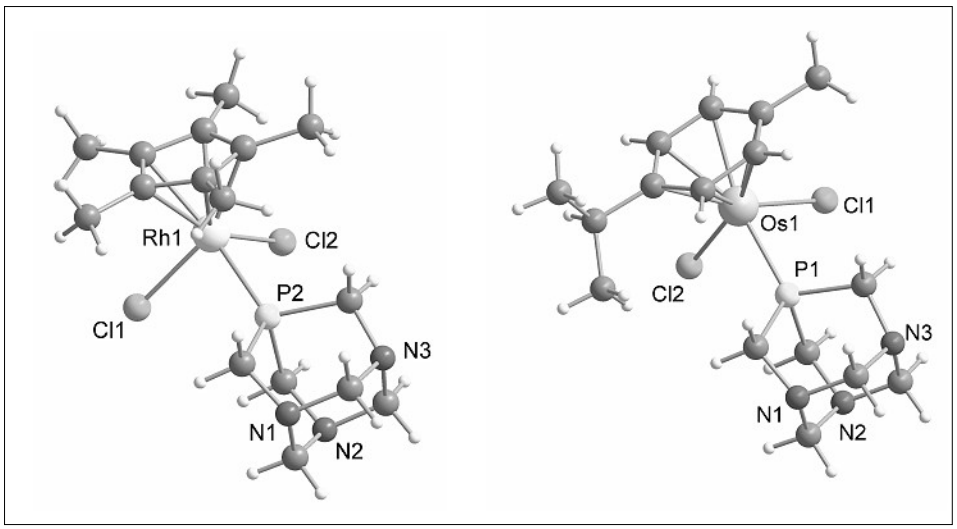

Fig. 4. Solid-state structure of (left) [Rh(η^5-C_5Me_5)Cl_2(pta)] and (right) [Os(η^6-cymene)Cl_2(pta)]; examples of RAPTA analogues based on other platinum group metals that exhibit different ligand exchange kinetics and redox properties

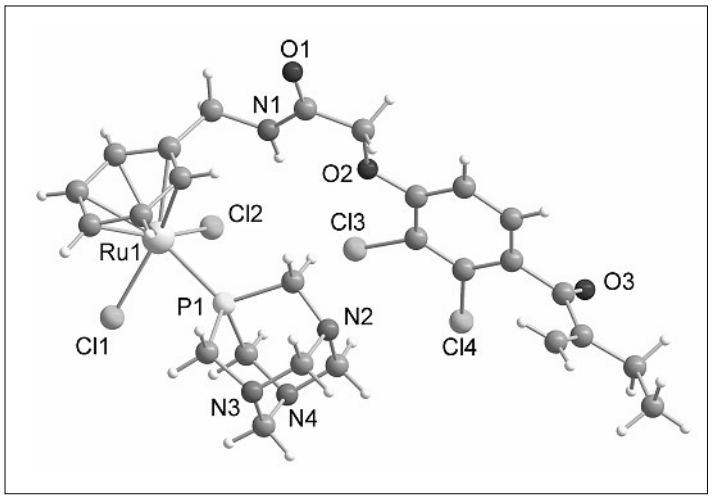

Fig. 5. Solid-state structure of ethaRAPTA-N1; an example of a RAPTA complex designed to overcome GST-mediated drug deactivation due to the presence of the ethacrynic acid (GST inhibiting) group

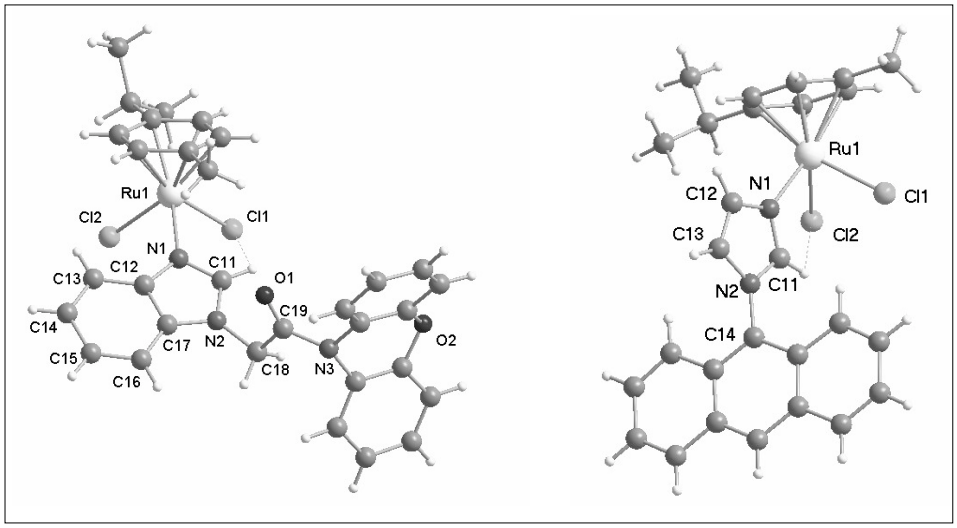

Fig. 6. Solid-state structures of two ruthenium(II)-arene compounds: (left) [Ru(η^6-*p*-cymene)Cl_2(phenoxbenzimide)] which contains a modified P-gp reversal agent as ligand and (right) [Ru(η^6-*p*-cymene)Cl_2(anthraimid)] – a structural analogue in which the O_2 atom has been replaced by a CH group

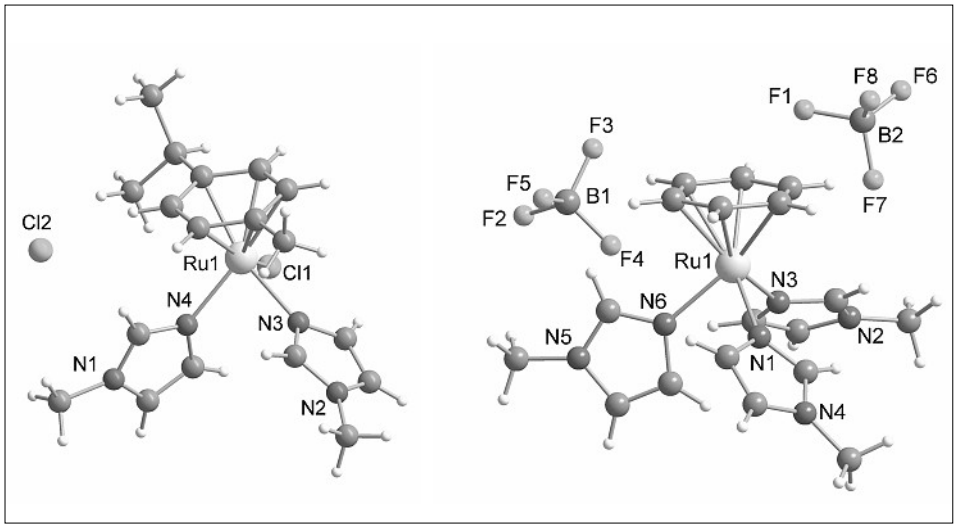

Fig. 7. Solid-state structure of (left) [Ru(η^6-benzene)$(mimid)_2$Cl]Cl and (right) [Ru(η^6-benzene)$(mimid)_3][BF_4]_2$ (mimid = N-methylimidazole); examples of hybrid RAPTA-NAMI antimetastatic drug candidates

of ruthenium in cell nuclei has already been described for the phase II clinical trial drug KP1019.[38]

Other Compounds

With a view to rationally develop other ruthenium-based antimetastatic agents, a series of ruthenium(II)–arene imidazole complexes have been prepared (Fig. 7), combining the unique structural aspects of NAMI-A and the ruthenium(II)–arene complexes described herein.[39] The complexes exhibit similar cytotoxicity with RAPTA complexes *in vitro* (TS/A, HBL-100) and several of the complexes exhibit selectivity towards cancer cells. We have also started to explore the potential effects of ruthenium–arene cluster compounds with Georg Süss-Fink and Bruno Thierren at the University of Neuchâtel and the latest results are very promising.[40]

Development of Techniques

In parallel to our research on the development of new drugs much of our efforts are oriented towards rationalising drug delivery and uptake mechanisms and determining drug targets and binding modes. In particular, we use a combination of separation and mass spectrometry techniques to achieve these goals – both electrospray ionisation and matrix assisted laser ablation.[41] Notably, we have developed a mass spectrometry method that reveals preferential binding of drugs to proteins from plasma[14] and whole cell mixtures[42] and have shown that binding site data can be obtained.[43] Currently we are expanding the method to human cells and potentially biopsy samples using 2D gel electrophoresis methods. While we have shown that specific compounds can inhibit certain enzymes (see above) it is clear that in a cell multiple interactions may occur with different biomolecules and the elucidation of these interactions should allow us to design more selective compounds.

Concluding Remarks

The notion of using ruthenium(II)–arene compounds as anticancer agents was first described by Tocher and co-workers, who coordinated the known anticancer agent 1-β-metronidazole to the ruthenium(II)–benzene fragment providing a compound with superior selective cytotoxicity than metronidazole itself.[44] Sadler has extensively studied ruthenium(II)–arene complexes with ethylenediamine and related ligands and their results are summarised elsewhere,[45] while Keppler's group developed Ru(II)–arene complexes with heterocyclic ligands.[46] A

series of ruthenium(II)–arene compounds with disulfoxide ligands have been tested *in vitro* for anticancer activity.[47] Our approach to combine aspects of both targeted and non-targeted chemotherapy into a single molecule based on a ruthenium(II)-arene frame has proven quite successful and it should not be too long before we know whether our lead compound, ethaRAPTA-N1 – Fig. 5, will reach clinical trials. Due to the very low toxicity of our compounds applications beyond cancer may also be possible.[48]

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