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Tuning the hydrophobicity of ruthenium(II)–arene (RAPTA) drugs to modify uptake, biomolecular interactions and efficacy†‡

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The antitumour activity of the organometallic ruthenium(II)–arene mixed phosphine complexes, [Ru(η^6-*p*-cymene)Cl(PTA)(PPh_3)]BF_4 **1b** and [Ru(η^6-$C_6H_5CH_2CH_2OH$)Cl(PTA)(PPh_3)]BF_4 **2b** (PTA = 1,3,5-triaza-7-phosphaadamantane), have been evaluated *in vitro* and compared to their RAPTA analogues, [Ru(η^6-*p*-cymene)Cl_2(PTA)] **1a** and [Ru(η^6-$C_6H_5CH_2CH_2OH$)Cl_2(PTA)] **2a**. The results show that the addition of the PPh_3 ligand to **2a** increases the cytotoxicity towards the TS/A adenocarcinoma cancer cells, which correlates with increased uptake, but also increases cytotoxicity to non-tumourigenic HBL-100 cells, thus decreasing selectivity. The decrease in selectivity has been correlated to increased DNA interactions relative to proteins, demonstrated by reactivity of the compounds with a 14-mer oligonucleotide and the model proteins ubiquitin and cytochrome-c.

Introduction

Platinum-based drugs are among the most effective clinical agents for the treatment of cancer with cisplatin, carboplatin, and oxaliplatin in widespread use.[1] These drugs exert their cytotoxicity by binding to DNA and all three compounds are believed to have similar molecular-level actions.[2] However, they present some clinical problems including acquired or intrinsic resistance that limits the spectrum of cancers that can be treated.[3] For most forms of disseminated cancer, nevertheless, no curative therapy is available, and the discovery and development of novel active chemotherapeutic agents remains crucial.

A plethora of non-platinum metal complexes have also been prepared and tested for anticancer activity.[4] Notably, ruthenium complexes have been found to be effective against cancers that cannot be treated with platinum drugs and it was also found that they exhibit a lower general toxicity compared to platinum compounds.[5,6] Two ruthenium compounds that have a different spectrum of activity to platinum drugs[7] are currently undergoing clinical evaluation.[8] One, NAMI-A (see Chart 1), shows high selectivity for solid tumour metastases and low toxicity at pharmacologically active doses and has successfully completed phase I clinical trials.[9] Metastasis control is associated with a series of biological activities that influence cell functions such as adhesion, motility, and invasion of tumour cells[10] involving f-actin condensation and a reduction of gelatinolytic capacity.[11]

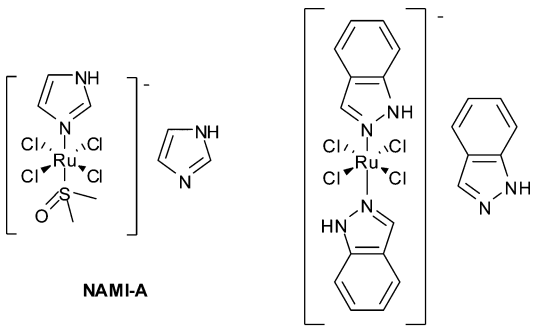

Chart 1 Clinically evaluated ruthenium-based anticancer drugs.

The other ruthenium compound, KP1019, was found to be active against colorectal tumours[12] and has successfully completed phase I clinical trials.[8a,13] Applying therapeutic doses of KP1019 neither caused significant toxic side effects in test animals nor in patients included in a clinical phase I study. Regarding the tumour-inhibiting properties of KP1019, 5 out of 6 evaluable patients treated with KP1019 showed disease stabilisation for up to 10 weeks.[8a]

More recently, increasing interest has focused on organometallic compounds,[14] specifically on ruthenium(II)–arene compounds which show excellent antiproliferative properties *in vitro* and *in vivo*.[6,15] Our research has focused on compounds with the general formula [Ru(η^6-arene)Cl_2(PTA)] (PTA = 1,3,5-triaza-7-phosphaadamantane), the prototype being [Ru(η^6-*p*-cymene)Cl_2(PTA)], termed RAPTA-C. Several structurally diverse RAPTA derivatives have been studied and their *in vitro* cytotoxicity has been evaluated.[16,17] The *in vivo* effect on the growth of lung metastases was also established for RAPTA-C and RAPTA-B, *i.e.* [Ru(η^6-benzene)Cl_2(PTA)].[17] The PTA ligand provides a degree of water solubility (dependent on the nature of the arene ligand) which facilitates administration and transport in the body. Some related ruthenium cyclopentadienyl

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complexes containing the PTA ligand, *viz.* $[RuCl(Cp')(PTA)_2]$ ($Cp' = C_5H_5$ or C_5Me_5) that show modest biological activity have been reported.[18] It has also been shown that the aromatic ring in RAPTA compounds may be replaced by the sulfur macrocycle 1,4,7-trithiacyclononane without changing the *in vitro* activity too significantly.[19] Moreover, some rhodium and osmium RAPTA analogues have been evaluated *in vitro* in HT29 colon carcinoma, A549 lung carcinoma and T47D breast carcinoma cell lines and again, activities are not too dissimilar from the ruthenium(II)–arene RAPTA complexes.[20]

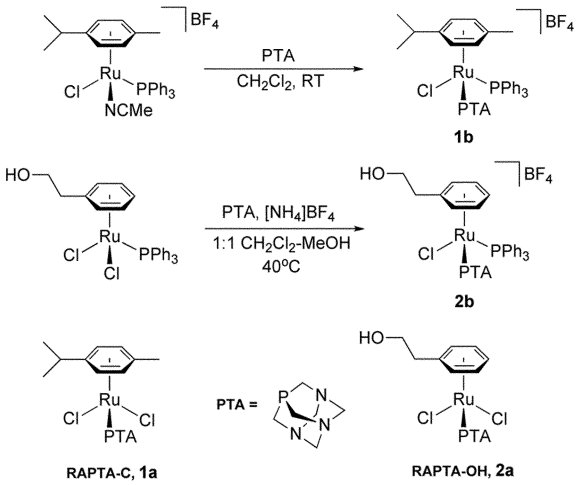
Peruzzini *et al.*[21] reported a series of water-soluble ruthenium complexes of general formula $[Ru(Cp)Cl(L)(L')]^{n+}$ ($L = PPh_3$, $L' =$ PTA, mPTA; $L = L' =$ PTA, mPTA; mPTA = *N*-methyl-1,3,5-triaza-7-phosphaadamantane). The interaction of the ruthenium complexes with DNA was found to be strongly dependent on the phosphine ligands present. Both methylation of the PTA or substitution of PTA by PPh_3 increases the reactivity with DNA.

Similar behaviour has also been observed for platinum thiosalicylate complexes, $[Pt(SC_6H_4CO_2)(L)]$ ($L =$ PTA or PPh_3),[22] where the PPh_3 derivative exhibits the highest antitumour activity toward leukemia P388 cells.[23] Some complexes containing PPh_3 such as *cis*-$[PtCl(PPh_3)_2(8\text{-MTT})]$, *cis*-$[Pt(PPh_3)_2(8\text{-MTT})_2]$, and *cis*-$[Pt(PPh_3)_2(8\text{-MTT})(8\text{-TTH})]$ are stronger inhibitors of the cisplatin-resistant SKOV3 cell line than analogous complexes containing PTA (8-TTH_2 = 8-thiotheophylline; 8-MTTH = 8-methylthiotheophylline).[24,25] The activity of the thiopurine platinum complexes on cisplatin-sensitive T2 cells is also strongly affected by the nature of the phosphine ligand, the complexes containing PPh_3 being significantly more active than those containing PTA.

In this paper we use the strategy described by Peruzzini to modify the hydrosolubility of the RAPTA compounds using PPh_3 in combination with PTA. In a previous study we attached arene ligands with hydrogen bonding substituents to the RAPTA framework as we hypothesised that it would increase the cytotoxicity of the RAPTA compounds by increasing their tendency to bind DNA/RNA *via* increased hydrogen bonding interactions.[16] While we were able to demonstrate increased reactivity with oligonucleotides the increased hydrophilicity of the compounds resulted in a dramatic decrease in uptake by the cells and correspondingly their cytotoxicity decreased. Thus, by incorporating a hydrophobic PPh_3 into the RAPTA framework we hope to reduce the hydrosolubility of the compounds with hydrogen bonding substituents and increase their uptake, and accordingly, their cytotoxicity.

Results and discussion

Substitution of the labile CH_3CN ligand in $[Ru(\eta^6\text{-}p\text{-cymene})Cl(CH_3CN)(PPh_3)]BF_4$ with PTA in CH_2Cl_2 at room temperature affords the mixed phosphine complex, $[Ru(\eta^6\text{-}p\text{-cymene})Cl(PTA)(PPh_3)]BF_4$ **1b**, in good yield (Scheme 1). The corresponding RAPTA complex bearing a hydroxyl-functionalised η^6-arene, $C_6H_5CH_2CH_2OH$, is prepared by ligand substitution of the chloride ligand in $[Ru(\eta^6\text{-}C_6H_5CH_2CH_2OH)Cl_2(PPh_3)]$, to give **2b** in high yield. Full synthetic, spectroscopic and analytical details are provided in the Experimental section, but of note, the ^{31}P NMR spectra of **1b** and **2b** exhibit a characteristic pair of doublets at *ca.* 30 ppm (PPh_3) and *ca.* −42 ppm (PTA), with a



Scheme 1 Synthesis of ruthenium(II)–arene mixed phosphine complexes **1b** and **2b**, and the structures of RAPTA-C **1a** and RAPTA-OH **2a**.

$^2J_{P\text{-}Ru\text{-}P}$ coupling constant of 54 Hz, corroborating the coordination of both phosphines to the ruthenium centre. Furthermore, the electrospray ionisation mass spectra (ESI-MS) of **1b** and **2b** display the desired parent ions with the expected isotopic abundances.

The structure of **1b** has been established in the solid state by single crystal X-ray diffraction and is depicted in Fig. 1. The structure exhibits the typical 'piano-stool' geometry around the ruthenium atom and has a close resemblance to that of the related bisphosphine complex, $[Ru(\eta^6\text{-}p\text{-cymene})Cl(PPh_3)_2]BF_4$ [Ru–P = 2.3649(6), 2.4042(6) Å],[26] other bisphosphine complexes,[27] and RAPTA-C **1a**,[28] although the Ru–PTA bond length is slightly elongated in comparison [2.3201(14) Å *vs.* 2.297(3) (average over two independent molecules) in RAPTA-C **1a**].

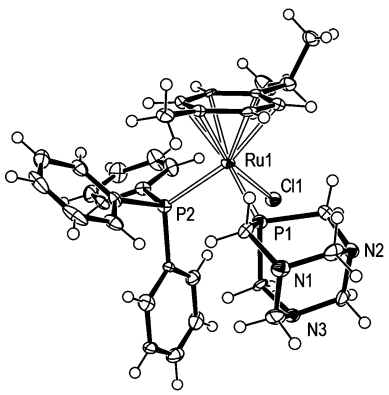


Fig. 1 ORTEP representation of **1b**; thermal ellipsoids are drawn at 50% probability level and the counter ion is omitted for clarity. Key bond lengths (Å) and angles (°): Ru1–Cl1, 2.4069(15); Ru1–P1, 2.3201(14); Ru1–P2, 2.3590(14); Ru1–C_{avg}, 2.26(4); Cl1–Ru1–P1, 82.78(5); Cl1–Ru1–P2, 90.35(5); P1–Ru1–P2, 95.32(5).

Cytotoxicity and uptake studies

The MTT test was carried out on compounds **1b** and **2b** using the tumourigenic TS/A and non-tumourigenic HBL-100 cell lines, previously used to evaluate the activity of other RAPTA compounds.[17] The effects of **1b** and **2b** on the growth of these cell lines were evaluated after 24, 48 and 72 h and the corresponding

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Table 1 IC_{50} values on the TS/A and HBL-100 cell lines after 72 h incubation

Compounds	IC_{50} (TS/A)/μM	IC_{50} (HBL-100)/μM
1a[17]	> 300	> 300
1b	> 300	37 ± 3
2a[17]	> 300	> 300
2b	124 ± 13	82 ± 9

Table 2 Ruthenium uptake after treatment of TS/A cells with 100 μM of drug for 24 h. Each number is the mean ± S.E. of an experiment made in triplicate

Compounds	Intracellular uptake/ μg 10^{-6} cells	Intracellular uptake/ $\times 10^{-4}$ M
1a[17]	0.12 ± 0.02	2.55 ± 0.06
1b	0.13 ± 0.03	1.71 ± 0.40
2a[17]	0.06 ± 0.01	1.33 ± 0.20
2b	0.20 ± 0.02	2.63 ± 0.23

IC_{50} values resulting from an average of two experiments are listed in Table 1.

As expected, the cytotoxicity of **2b** is greatest in the tumour cells, the other three compounds not showing any significant cytotoxicity up to 300 μM. The increased cytotoxicity of **2b**, at least relative to **2a**, corresponds well with increased drug uptake (Table 2), being *ca.* 3 fold higher. In contrast, the addition of the PPh_3 ligand to RAPTA-C has only a minor effect on cytotoxicity probably due to the poor solubility of **1b** in the medium.

Importantly, the presence of the PPh_3 ligand decreases the selectivity of the compounds towards the tumour cells as evidenced by the lower IC_{50} values of **1b** and **2b** in the non-tumourigenic HBL-100 cells (used to model healthy cells) relative to **1a** and **2a**. The higher hydrophobicity of the ruthenium(II)–arene compounds with the PPh_3 ligands would appear to have the effect of increasing the general cytotoxicity of the complexes, which may also be connected to differences in reactivity with various biomolecules (see later).

Comparative interactions with model biomolecules

The traditional target for metal-based anticancer drugs is DNA, and damage of DNA is implicated in the activity of several important metal-based anticancer drugs.[29] Accordingly, the reactivity of **1b**, **2b** and their precursors RAPTA-C **1a** and RAPTA-OH **2a** with the 14-mer 5′-ATACATGGTACATA-3′, was studied by electrospray ionisation mass spectrometry (ESI-MS). Essentially, the compounds were incubated in water with the 14-mer at 37 °C for 72 h in a 5 : 1 (ruthenium : 14-mer) ratio and the products analysed by ESI-MS. Following incubation, the solutions were diluted with DMBA (dimethylbenzylamine), and injected into the mass spectrometer. The negative ion ESI deconvolution mass spectrum for the incubation with **2b** is shown in Fig. 2 as an illustrative example, complete data are summarised in Table 3.

Inspection of these data reveals a number of trends. First, **2b** is able to bind to the oligonucleotide as shown by the peak at *m/z* 4790 (relative abundance 100%) which corresponds to [14-mer-Ru(PTA)(PPh_3)] (no interaction is detected with **1b**, presumably due to its insolubility in the incubation medium). It is noteworthy that no unreacted 14-mer is present in the spectrum of **2b** indicating

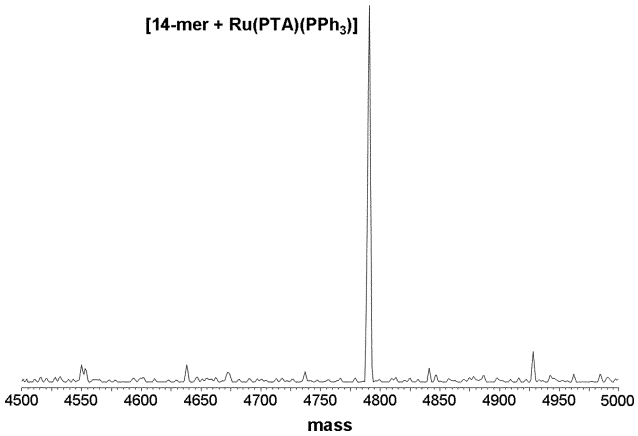

Fig. 2 Negative ion ESI mass spectrum (deconvoluted) of a 5 : 1 incubation mixture of **2b** + 14-mer in water (37 °C, 72 h incubation).

Table 3 Species observed after deconvolution of the ESI spectra from the incubation of the compounds with the 14-mer 5′-ATACATGGTACATA-3′ (ratio 5 : 1)[a]

Compounds	Adducts
1a	[14-mer + Ru(PTA) + Ru(η⁶-*p*-cymene)(PTA)] [14-mer + Ru(PTA)] (92%) [14-mer + Ru(η⁶-*p*-cymene) (PTA)] (22%) 14-mer (18%)
1b	14-mer
2a	[14-mer + 2Ru(PTA)] [14-mer + 2Ru($C_6H_5CH_2CH_2OH$)(PTA)] (80%) [14-mer + 2Ru(PTA) + 2Ru($C_6H_5CH_2CH_2OH$)(PTA) + Cl^-] (80%) [14-mer + Ru($C_6H_5CH_2CH_2OH$)(PTA) + Ru($C_6H_5CH_2CH_2OH$)] (55%) [14-mer + 2Ru(PTA) + Ru($C_6H_5CH_2CH_2OH$)(PTA)] (45%)
2b	[14-mer + Ru(PTA)(PPh_3)]

[a] Mass of the 14-mer is 4271. The percentage in parentheses corresponds to the relative intensity (first species at 100%).

the high affinity of **2b** towards nucleobase targets. In comparison, **1a** and **2a** are less reactive. It appears that as a first contact between the ruthenium(II)–arene compound and the oligonucleotide, the structure of the arene ligand is important to favour an interaction, as demonstrated elsewhere.[30] Most probably, the binding of compound takes place initially *via* the rapid hydrolysis of the chloride in solution, followed by substitution of the water ligands for the 14-mer. Next, the arene ligand may be lost to give the possibility to the oligonucleotide to occupy the surroundings of the ruthenium centre. As postulated, these data indicate that hydrogen bonding between the arene ligand in **2b** and the DNA will favour a first contact.

Ubiquitin (Ub) and cytochrome-c (Cyt-C) have been used as model proteins to study the binding of metal complexes to proteins using mass spectrometry with both clinically established and developmental drugs. It was reported that the reactivity of cisplatin, transplatin, carboplatin, and oxaliplatin toward cytochrome-c is quite similar.[31] The reaction of the ruthenium–arene complex, [(η⁶-biphenyl)Ru(en)Cl][PF_6], toward cytochrome-c has been studied by mass spectrometric and NMR methods and it was

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found that when incubating Cyt-C with a ten-fold excess of [(η⁶-bip)Ru(en)Cl][PF_6] in water and in TEAA buffer at pH 8.7 and 7.6, respectively, resulted in the formation of a monoruthenated species.[32] RAPTA compounds have also been shown to bind to Cyt-C and lysozyme with His33 was proposed as a major interaction site in the former.[33] Reactions of ubiquitin with platinum complexes including cisplatin, transplatin, and asymmetric *trans*-Pt complexes have also been reported.[34] Cisplatin was found to form 1 : 1 and 1 : 2 adducts, while transplatin forms mainly $Pt(NH_3)_2ClUb$. The asymmetric Pt(II) complexes were reported to coordinate exclusively to Met1 of ubiquitin which is also supposed to be the main target for cisplatin but not for transplatin.

Similarly, complexes **1a**, **1b**, **2a** and **2b** were incubated with Ub or Cyt-C at molar ratios of 5 : 1 and analysed by offline nanoelectrospray ionisation-ion trap-mass spectroscopy (nESI-IT-MS).

Ub reacts with **1a** to form the monoruthenated adduct Ru(η⁶-*p*-cymene)–Ub with a mass of 8807 Da after 5 h incubation. The corresponding peak in the deconvoluted mass spectrum was the most abundant signal, followed by the signal of Ub (8575 Da, 52%). After incubation for 1 day the signal for Ub was no longer present. As for the 14-mer, **1b** did not react with Ub. The most abundant peak in the mass spectrum corresponded to the complex [Ru(η⁶-*p*-cymene)Cl(PPh_3)(PTA)]⁺. In contrast to **1a**, incubation of **2a** with ubiquitin for 5 h affords a mass spectrum in which the most abundant peak corresponds to Ub with peaks of lower relative intensity at 8706 Da (54%) and 8785 Da (19%) that correspond to a RuCl–Ub adduct and the Ru(η⁶-arene)–Ub conjugate. The analysis after 1 day of incubation yielded the trifunctional Ru(η⁶-arene)–Ub species as the peak with the highest relative intensity (a considerable amount of Ub was still detected, *ca.* 52%). After 2 days, a spectrum with only a Ru(η⁶-arene)–Ub adduct was observed. The reaction of **2b** was found to proceed more slowly than the parent compound **2a**, but under the applied conditions forms the same adducts, although the Ub peak is still the most abundant protein peak in the mass spectrum. Note that even after 6 days of incubation, all the protein peaks are minor compared to the signal of [Ru(η⁶-arene)Cl(PPh_3)(PTA)]⁺.

For comparison purposes, analysis of the incubation solutions was also conducted using nanoelectrospray ionisation-quadrupole time of flight-mass spectrometry (nESI-QToF-MS). The reaction mixtures were pre-concentrated on a C_{18} column and, by reversing the flow, the samples were introduced into the mass spectrometer. However, in comparison to the analyses performed on the ion trap system, the formation of a lower number of adducts with lower intensities were observed. Ub (8562 Da) was found to form with **1a** and **2a** trifunctional adducts of the type Ru(η⁶-arene)–Ub (8795 and 8783 Da for **1a** and **2a**, respectively) and to a minor degree of bifunctional Ru(η⁶-arene)(PTA)–Ub (for **2a** the time course of the reaction is presented in Fig. 3; a similar reactivity was found for **1a**). In contrast, the compounds **1b** and **2b** behaved quite differently: while **1b** did not show any reaction with ubiquitin; **2b** reacts with Ub to form Ru(PPh_3)(PTA)–Ub (9080 Da, 5%) after 20 h of incubation. The peaks of the complex cations [Ru(η⁶-arene)Cl(PPh_3)(PTA)]⁺ (*m*/*z* 689.8 and 677.8 for **1b** and **2b**, respectively) were the most abundant, and the binding of **2b** toward Ub was found to be slower than of the neutral compound **2a**. After incubation for 20 h, for **1a** and **2a** the same Ru(η⁶-arene)–

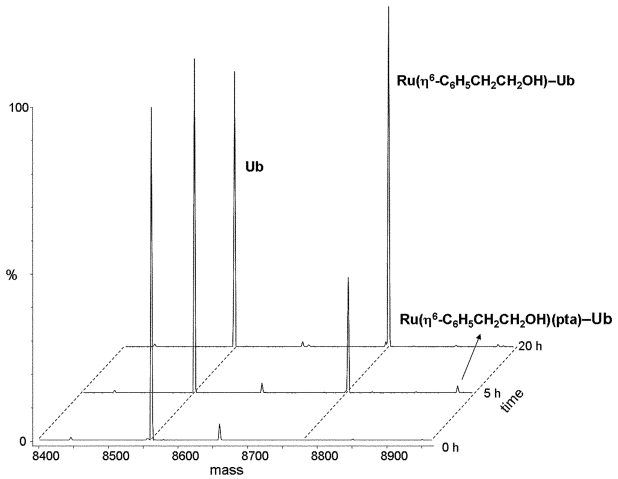

Fig. 3 Time course of the reaction of **2a** with Ub followed by nESI-QToF-MS; Ub and **2a** were incubated at a molar ratio of 1 : 5 at 37 °C.

Ub conjugates were found as in the IT mode but at a significantly lower intensity.

The reaction of Cyt-C with the Ru complexes was followed by nESI-IT-MS. The neutral complexes **1a** and **2a** were found to form with Cyt-C the same adducts as with Ub, *i.e.*, Ru(η⁶-arene)–Cyt-C, but the reaction kinetics were markedly decreased. The complexes with the PPh_3 ligands did not form any detectable adducts even after 6 days of incubation. These data are summarised in Table 4.

Table 4 Species observed from the incubation mixture of the ruthenium compounds and Cyt-C in water after 5 h incubation at 37 °C

Compound	Adducts
1a	[Cyt-C + Ru(η⁶-*p*-cymene)]
1b	None observed
2a	[Cyt-C + Ru(η⁶-$C_6H_5CH_2CH_2OH$)]
2b	None observed

The differences in reaction kinetics for the different proteins might be explained by the availability of metal-binding sites on the surface of proteins.[35] Although Cyt-C contains twice the number of methionines, three more histidines and two cysteine residues than Ub, reactivity towards Cyt-C is slower than that of Ub. Met1 and His68 of Ub are easier to access than the potential metal-binding sites in Cyt-C as they are more exposed on the protein surface (Fig. 4). What is clear, however, is that the more

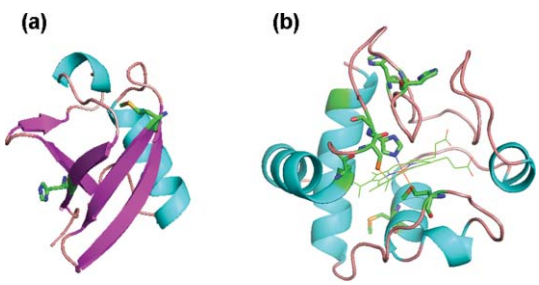

Fig. 4 Molecular structures of (left) ubiquitin[36] and (right) cytochrome-c[37] with the His, Met and Cys residues highlighted as stick graphics (PyMOL v0.98, (c) DeLano Scientific LLC).

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hydrophobic (and sterically demanding) complexes **1b** and **2b** are less reactive towards both proteins than **1a** and **2a**. Moreover, selective (preferential) interactions are also observed.

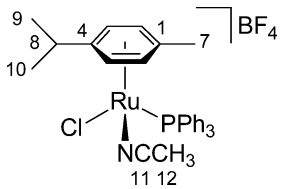
Concluding remarks

From an overview of the data presented herein, it would appear that as the structure of RAPTA compounds are modified in such a way that DNA binding interactions are favoured over interactions with proteins, the general toxicity of the compounds increases. It is possible that this trend could be a general phenomenon although further experiments of different classes of anticancer compounds would be required to substantiate this view. It would certainly be interesting to evaluate the platinum compounds described in the Introduction that were modified in a similar manner on non-tumourigenic cells. Nevertheless, this work demonstrates the importance of finding targets other than DNA for the design of new putative anticancer reagents that will exhibit high selectivity and thus reduced general side-effects.

Experimental

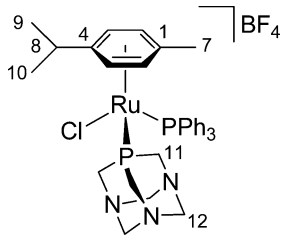
Reactions were carried out under a nitrogen atmosphere and solvents were purged with nitrogen before use. 1,3,5-Triaza-7-phosphaadamantane (PTA),[38] [Ru(η⁶-*p*-cymene)Cl_2(PPh_3)],[39] and [Ru(η⁶-$C_6H_5CH_2CH_2OH$)Cl_2]$_2$[40] were prepared as described elsewhere. All other chemicals are commercial products and were used as received. NMR spectra were recorded with a Bruker Avance 400 spectrometer at room temperature, unless otherwise stated. Chemical shifts are given in ppm and coupling constants (J) in Hz. ESI mass spectra for characterisation of synthetic compounds were recorded on a Thermo Finnigan LCQ DECA XPPlus according to a literature procedure[41] and microanalyses were performed at the EPFL.

[Ru(η⁶-*p*-cymene)Cl(CH_3CN)(PPh_3)]BF_4



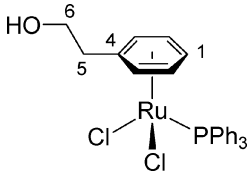
A suspension of [Ru(η⁶-*p*-cymene)Cl_2(PPh_3)] (5.22 g, 9.18 mmol) and NH_4BF_4 (1.25 g, 11.04 mmol) in CH_3CN (180 ml) was stirred at RT for 4 days. The solvent was then removed and the residue extracted with CH_2Cl_2 through Celite. The product was precipitated following concentration and addition of pentane. Yield: 4.82 g (80%) as a yellow powder. NMR data are in agreement with the analogous PF_6 complex.[42] ^{1}H NMR ($CDCl_3$): δ 7.46–7.68 (m, 15H, P*Ph*$_3$), 6.33 (d, $^3J_{HH}$ = 6.0, 1H, H^5), 5.92 (d, $^3J_{HH}$ = 5.9, 1H, H^3), 5.38 (d, $^3J_{HH}$ = 5.9, 1H, H^2), 4.70 (d, $^3J_{HH}$ = 6.2, 1H, H^6), 3.11 (sept, $^3J_{HH}$ = 6.8, 1H, H^8), 1.99 (s, 3H, H^{12}), 1.74 (s, 3H, H^7), 1.38 (d, $^3J_{HH}$ = 7, 3H, H^9), 1.36 (d, $^3J_{HH}$ = 7, 3H, H^{10}). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 35.7 (s, Ru*P*Ph_3). ^{11}B{1H} NMR ($CDCl_3$) δ −0.8 (pent, $^1J_{BF}$ = 1, *B*F_4). ESI-MS (CH_2Cl_2) positive ion: m/z, 533 (23%) [M − CH_3CN]$^+$, 574 [M]$^+$; negative ion: m/z, 87 [BF_4]$^-$. Calcd for $C_{30}H_{32}BClF_4NPRu$ (660.89 g mol^{-1})·0.6($CH_3CH_2CH_2CH_2CH_3$): C, 56.29; H, 5.61; N, 1.99. Found: C, 56.64; H, 5.28; N, 2.31.

[Ru(η⁶-*p*-cymene)Cl(PTA)(PPh_3)]BF_4 1b



A solution of [Ru(η⁶-*p*-cymene)Cl(CH_3CN)(PPh_3)]BF_4 (0.50 g, 0.76 mmol) and PTA (0.15 g, 0.99 mmol) in CH_2Cl_2 (25 ml) was stirred at RT for 90 min. The solution was then washed with water (2 × 50 ml), dried with Na_2SO_4 and the product was precipitated by the addition of pentane. Yield: 0.44 g (75%) as a yellow microcrystalline powder. ^{1}H NMR ($CDCl_3$): δ 7.48–7.70 (m, 15H, P*Ph*$_3$), 6.69 (dd, $^3J_{HH}$ = 6.2, $^3J_{PH}$ = 5, 1H, H^6), 5.94 (d, $^3J_{HH}$ = 5.8, 1H, H^5), 5.80 (d, $^3J_{HH}$ = 6.1, 1H, H^3), 5.13 (dd, $^3J_{HH}$ = 6.4, $^3J_{PH}$ = 5, 1H, H^2), 4.45 (d, $^2J_{HH}$ = 13.1, 3H, H^{12}), 4.36 (d, $^2J_{HH}$ = 13.2, 3H, H$^{12'}$), 4.26 (d, $^2J_{HH}$ = 14.3, 3H, H^{11}), 3.83 (d, $^2J_{HH}$ = 14.2, 3H, H$^{11'}$), 2.71 (sept, $^3J_{HH}$ = 6.9, 1H, H^8), 1.29 (d, $^3J_{HH}$ = 7, 3H, H^9), 1.27 (d, $^3J_{HH}$ = 7, 3H, H^{10}). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 134.4 (d, $^2J_{PC}$ = 10, P*Ph*$_3$), 133.1 (d, $^1J_{PC}$ = 48, P*Ph*$_3$), 131.6 (d, $^4J_{PC}$ = 2, P*Ph*$_3$), 128.9 (d, $^3J_{PC}$ = 10, P*Ph*$_3$), 125.2 (d, $^2J_{PC}$ = 4, C^4), 101.7 (s, C^1), 97.9 (d, $^2J_{PC}$ = 4, C^2), 93.8 (d, $^2J_{PC}$ = 8, C^3), 91.0 (d, $^2J_{PC}$ = 2, C^6), 88.2 (d, $^2J_{PC}$ = 8, C^5), 72.5 (d, $^3J_{PC}$ = 8, C^{12}), 53.7 (d, $^1J_{PC}$ = 15, C^{11}), 31.0 (s, C^8), 22.0 (s, C$^{9/10}$), 21.5 (s, C$^{10/9}$), 17.7 (s, C^7). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 29.9 (d, $^2J_{PP}$ = 54, 1P, Ru*P*Ph_3), −42.8 (d, $^2J_{PP}$ = 54, 1P, Ru*PTA*). ^{11}B{1H} NMR ($CDCl_3$) δ −0.7 (pent, $^1J_{BF}$ = 1, *B*F_4). ESI–MS (CH_2Cl_2) positive ion: m/z, 690 [M]$^+$; negative ion: m/z, 87 [BF_4]$^-$. Calcd for $C_{34}H_{41}BClF_4N_3P_2Ru$ (776.99 g mol^{-1}): C, 52.56; H, 5.32; N, 5.41. Found: C, 52.31; H, 5.36; N, 5.33.

[Ru(η⁶-$C_6H_5CH_2CH_2OH$)Cl_2(PPh_3)]



A suspension of [Ru(η⁶-$C_6H_5CH_2CH_2OH$)Cl_2]$_2$ (0.80 g, 1.36 mmol) and PPh_3 (0.89, 3.39 mmol) in 2 : 1 CH_2Cl_2–MeOH (30 ml) was stirred at RT for 90 min forming a dark homogeneous phase. The solution was then slowly concentrated *in vacuo* resulting in precipitation of the product as an orange–red solid. Yield: 1.07 g (71%). ^{1}H NMR ($CDCl_3$): δ 7.68–7.83 (m, 6H, *ortho*-P*Ph*$_3$), 7.32–7.50 (m, 9H, P*Ph*$_3$), 5.64 (d, $^3J_{HH}$ = 5.8, 2H, H^3), 5.05 (dd, $^3J_{HH}$ = 5, $^3J_{HH}$ = 5, 2H, H^2), 3.88 (dt, $^3J_{HH}$ = 5, $^3J_{PH}$ = 3, 1H, H^1), 4.14 (t, $^3J_{HH}$ = 5.4, 2H, H^6), 2.92 (t, $^3J_{HH}$ = 5.1, 2H, H^5), 2.86 (br, 1H, O*H*). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 134.2 (d, $^2J_{PC}$ = 10, P*Ph*$_3$), 130.5 (d, $^4J_{PC}$ = 2, P*Ph*$_3$), 128.2 (d, $^3J_{PC}$ = 10, P*Ph*$_3$), 108.7 (d, $^2J_{PC}$ = 9, C^4), 91.7 (d, $^2J_{PC}$ = 5, C^3), 86.0 (br, C^2), 82.8 (s, C^1), 60.7 (s, C^6), 35.4 (s, C^5). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 27.8

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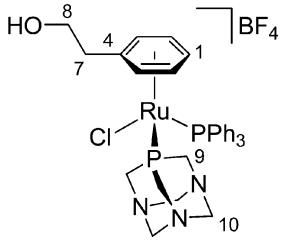
(s, Ru*P*Ph$_3$). Calcd for $C_{26}H_{25}Cl_2OPRu$ (556.43 g mol^{-1}): C, 56.12; H, 4.53. Found: C, 55.76; H, 4.57.

[Ru(η^6-($C_6H_5CH_2CH_2OH$)Cl(PTA)(PPh_3)]BF_4 2b



A solution of [Ru(η^6-$C_6H_5CH_2CH_2OH$)Cl_2(PPh_3)] (0.20 g, 0.36 mmol), PTA (0.085 g, 0.54 mmol) and NH_4BF_4 (0.057 g, 0.54 mmol) in 1 : 1 CH_2Cl_2–MeOH (20 ml) was stirred at 40 °C for 1 h. The solvent was removed *in vacuo* and the residue suspended in CH_2Cl_2 (50 ml), then washed with water (2 × 50 ml). The aqueous phase was extracted with CH_2Cl_2 (3 × 50 ml) and the combined organic phases dried with Na_2SO_4. Concentration and addition of excess hexane gave the product as a yellow powder. Yield: 0.24 g (87%). ^{1}H NMR ($CDCl_3$): δ 7.45–7.60 (m, 15H, P*Ph*$_3$), 6.44–6.57 (m, 1H, H^2), 6.22 (d, $^3J_{HH}$ = 5.5, 1H, H^3), 5.05 (d, $^3J_{HH}$ = 6.2, 1H, H^5), 5.41–5.51 (m, 1H, H^6), 4.39–4.48 (m, 1H, H^1), 4.31 (s, 6H, H^{10}), 4.18 (d, $^2J_{HH}$ = 14.5, 3H, H^9), 3.80–4.04 (m, 2H, H^8), 3.92 (d, $^2J_{HH}$ = 13.2, 3H, H$^{9'}$), 2.67 (t, $^3J_{HH}$ = 5, 2H, H^7), 2.29 (br, 1H, O*H*). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 134.3 (d, $^2J_{PC}$ = 10, P*Ph*$_3$), 133.3 (d, $^1J_{PC}$ = 49, PPh_3), 131.7 (d, $^4J_{PC}$ = 2, P*Ph*$_3$), 129.0 (d, $^3J_{PC}$ = 10, P*Ph*$_3$), 118.2 (d, $^2J_{PC}$ = 5, C^4), 97.8 (d, $^2J_{PC}$ = 8, C^5), 97.5 (d, $^2J_{PC}$ = 4, C^6), 91.7 (d, $^2J_{PC}$ = 9, C^3), 89.6 (br, C^2), 85.0 (s, C^1), 72.4 (d, $^3J_{PC}$ = 8, C^{10}), 60.1 (s, C^8), 53.6 (d, $^1J_{PC}$ = 15.4, C^9), 35.6 (s, C^7). ^{31}P{^{1}H} NMR ($CDCl_3$) δ 32.0 (d, $^2J_{PP}$ = 53, 1P, Ru*P*Ph$_3$), −42.0 (d, $^2J_{PP}$ = 54, 1P, Ru*PTA*). ^{11}B{1H} NMR ($CDCl_3$) δ −0.8 (pent, $^1J_{BF}$ = 1, *B*F$_4$). ESI–MS (CH_2Cl_2) positive ion: *m*/*z*, 678 [M]$^+$; negative ion: *m*/*z*, 87 [BF_4]$^-$. Calcd for $C_{32}H_{37}BClF_4N_3OP_2Ru$ (764.94 g mol^{-1})·1/2(H_2O): C, 49.66; H, 4.95; N, 5.43. Found: C, 49.55; H, 5.14; N, 5.25.

Crystallography†

Crystals suitable for X-ray diffraction were obtained from slow diffusion of pentane into a $CHCl_3$ solution of **1b**. Relevant details about the structure refinements are given in Table 5, and selected geometrical parameters are included in the captions of Fig. 1. Data collection was performed on a KUMA CCD diffractometer system using graphite monochromated Mo Kα radiation (0.71073 Å) and a low-temperature device. Data reduction was performed using CrysAlis RED.[43] Structures were solved using SIR97,[44] and refined (full-matrix least squares on F^2) using SHELXTL.[45] All non-hydrogen atoms were refined anisotropically, with hydrogen atoms placed in calculated positions using the riding model. The graphical representation was made with ORTEP3.[46]

Cell growth inhibition effects on TS/A adenocarcinoma cells

TS/A murine adenocarcinoma cell line, initially obtained from Dr G. Forni (CNR, Centro di Immunogenetica ed Oncologia Sperimentale, Torino, Italy) belong to the tumour cell lines panel of the Callerio Foundation and is stored in liquid nitrogen. Cells were cultured according to a standard procedure,[47] and maintained in RPMI-1640 medium (EuroClone, Wetherby, UK) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Milano, Italy), 2 mM L-glutamine (EuroClone, Wetherby, UK) and 50 μg ml^{-1} gentamycin sulfate solution (EuroClone, Wetherby, UK). The cell line was kept in an incubator with 5% CO_2 and 100% relative humidity at 37 °C. Cells from a confluent monolayer were removed from flasks by a trypsin–EDTA solution (EuroClone, Wetherby, UK).

HBL-100 non-tumorigenic human breast cells, obtained from ATCC (American Type Culture Collection), were maintained in McCoy's 5A medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 UI ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (EuroClone, Whetherby, UK) in a humidified atmosphere with 5% CO_2 at 37 °C.

Cell viability was determined by the trypan blue dye exclusion test. For experimental purposes, the cells were sown in multiwell cell culture plastic plates (Corning Costar Italia, Milano, Italy). Cell growth was determined by the MTT viability test.[48] Cells were sown on 96-well plates and after 24 h were incubated with the appropriate compound, prepared by dissolving in a medium containing 5% of serum for 24, 48 and 72 h. Solutions of the substances were prepared by diluting a freshly prepared stock solution of compound **2b** in water (10^{-2} M) and compound **1b** in DMSO (10^{-2} M). Maximum DMSO concentration in the cells was 1% v/v. Analysis was performed at the end of the incubation time. Briefly, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dissolved in PBS (5 mg ml^{-1}) was added (10 μl per 100 μl of medium) to all wells and the plates were then incubated at 37 °C with 5% CO_2 and 100% relative humidity for 4 h. After this time, the medium was discarded and 100 μl of DMSO (Sigma, St. Louis, MO, USA) were added to each well according to the method of Alley *et al.*[49] Optical density was measured at 570 nm on a SpectraCount Packard (Meriden, CT) instrument.

Table 5 Crystallographic data for **1b**

Formula	$C_{34}H_{41}BClF_4N_3P_2Ru$
M	776.97
T/K	140(2)
Cryst. syst.	Monoclinic
Space group	*P*2(1)/*c*
a/Å	13.6698(10)
b/Å	14.4658(9)
c/Å	17.1533(6)
α/°	
β/°	99.576(5)
δ/°	
V/Å^3	3344.7(3)
Z	4
Density/g cm^{-3}	1.543
μ/mm^{-1}	0.697
θ range/°	2.98 ≤ *θ* ≤ 25.02
Measured reflns	19880
Unique reflns	5666 [R_{int} = 0.0773]
No. data/restr./param.	5666/0/418
*R*1, *wR*2 [*I* > 2*σ*(*I*)][a]	0.0384, 0.0571
GoF[b]	0.774

[a] $R1 = \Sigma \| F_o | - | F_c \| / \Sigma | F_o |$, $wR2 = \{\Sigma[w(F_o^2 - F_c^2)^2] / \Sigma[w(F_o^2)^2]\}^{1/2}$. [b] GoF = $\{\Sigma[w(F_o^2 - F_c^2)^2]/(n-p)\}^{1/2}$ where *n* is the number of data and *p* is the number of parameters refined.

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Determination of intracellular ruthenium

Ruthenium cell uptake was determined by atomic absorption spectroscopy (AAS) on samples processed using the procedure of Tamura and Arai with slight modifications.[50] For each complex tested, a 6-well plate was prepared by seeding 1.25×10^5 TS/A cells in 3 ml of complete medium with 5% FBS to each experimental and control well. The plate was incubated for 24 h at 37 °C. The wells were then washed three times with PBS. Control wells were filled with 3 ml of complete medium and experimental wells with 3 ml of a 100 μM solution of ruthenium compounds prepared in complete medium. The plate was incubated for 24 h at 37 °C before the cells were collected and counted with the trypan blue exclusion test and the intracellular concentration of ruthenium was determined. After this treatment, the cells were dried in Nalgene® cryogenic vials (a first drying step was performed overnight at 80 °C and a second step at 105 °C until the samples reached a constant weight). The dried cells were decomposed by the addition of an aliquot of tetramethylammonium hydroxide (25% in water) (Aldrich Chimica, Gallarate, Milano, Italy) and of milliQ water at a ratio of 1 : 1 directly in each vial at room temperature under shaking. Final volumes were adjusted to 1 ml with milliQ water. The concentration of ruthenium in TS/A tumour cells was measured in triplicate by flameless atomic absorption spectroscopy (AAS) using a Zeeman graphite tube atomizer, model SpectrAA-300, supplied with a specific ruthenium emission lamp (hollow cathode lamp P/N 56–101447-00; Varian, Mulgrave, Victoria, Australia). Quantification of ruthenium was carried out in 10 μl samples at 349.9 nm with an atomising temperature of 2500 °C, using argon as carrier gas at a flow rate of 3.0 l min^{-1} (for further details concerning the furnace parameter settings, see ref. 51). Before each analysis, a five-point calibration curve was obtained to check the range of linearity using ruthenium custom-grade standard 998 mg ml^{-1} (Inorganic Ventures, Lakewood, N.J.).

Oligonucleotide binding

The 14-mer oligonucleotide (5′-ATACATGGTACATA-3′) was obtained from MWG biotech AG (Ebersberg, Germany) and the concentration was taken to be 100 μM as specified by the supplier. The samples were prepared in a ratio of 1 : 5 by mixing the 14-mer (50 μM) with an aqueous solution of the ruthenium complex (250 μM). In order to dissolve the compound **1b**, DMSO was added with a final DMSO concentration of 1% in H_2O. The samples were maintained at 37 °C for 72 h with vigorous shaking. The ESI measurements were performed on a Micromass Q-Tof Ultima. When necessary the samples were desalted through a Microcon® centrifugal filter device (Millipore) directly after the incubation. Then the solutions were diluted twice with DMBA (25 μM; dimethylbenzylamine) and injected into the mass spectrometer. The spectra were recorded in negative mode and before every series of measurements the spectrometer was calibrated with H_3PO_4. The source temperature was set at 373 K and the cone voltage to 35 keV, with a mass range from 400 to 2000. The acquisition and the deconvolution of data were performed on a Windows XP PC system using Mass Lynx (version 4.0) and the Max Ent Electrospray software algorithm.

Protein binding studies

Ubiquitin (from bovine red blood cells, min. 90%) and cytochrome-c (from horse heart, 96%) were purchased from Sigma (Steinheim, Germany). Formic acid (98–100%), acetonitrile (HPLC gradient grade), and water (HPLC gradient grade) came from Merck (Darmstadt, Germany), Fisher Scientific (Loughborough, UK), and Acros Organics (Geel, Belgium), respectively.

Complexes (**1a**, **1b**, **2a** and **2b**) were incubated at molar ratios of 5 : 1 with the proteins in aqueous solution at 37 °C yielding a concentration of 10 μM protein. In order to dissolve the compound **1b**, DMSO was added with a final DMSO concentration of 1% in H_2O. Before nESI-IT-MS analysis, the incubation solutions were mixed at a ratio of 1 : 3 with ACN : H_2O : formic acid (68 : 32 : 0.7) and the samples were analysed immediately after mixing (after 5 h, 1 day, 2 days, 3 days, and 6 days incubation).

nESI-IT-MS. Analyses were performed on a Thermo Finnigan LCQ Deca XP Plus quadrupole ion-trap instrument in positive ion mode. The capillary temperature was set at 180 °C and the source voltage to 1.51 kV, with a mass range from 300 to 2000. The acquisition was performed with Tune Plus 1.3 SR1 program (Thermo Finnigan) and the deconvolution of data were performed with Bioworks Browser 3.0 (Thermo Finnigan) on a Windows 2000 Prof. SR4 PC system using Biomass Calculation and Deconvolution software.

nESI-QToF-MS. The samples were introduced into the mass spectrometer by means of a Waters modular CapLC system. Samples were loaded onto a C_{18} cartridge (Symmetry 300™, C_{18}, 5 μm NanoEase™) and desalted with 0.1% HCOOH for 3 min at a flow rate of 30 μl min^{-1}. The 10-port valve was then switched such that the samples were sprayed directly into the mass spectrometer. The electrospray potential (*ca.* 2 kV) was applied to the liquid *via* a low dead volume stainless steel union. A small amount (*ca.* 2.5 psi) of nebulising gas was introduced around the spray tip to aid the electrospray process. Data were acquired using a Micromass Q-Tof Ultima mass spectrometer, equipped with Z-spray nanoflow electrospray ion source. The mass spectrometer was operated in the positive-ion mode with a source temperature of 80 °C and a counter current gas flow rate of 40 l h^{-1}. The instrument was calibrated with phosphoric acid in the range of 100–2000 m/z. All data were processed using MassLynx version 4.0 software using Max Ent Electrospray software algorithm.

Acknowledgements

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