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# Strategy To Tether Organometallic Ruthenium–Arene Anticancer Compounds to Recombinant Human Serum Albumin

**Wee Han Ang,[†] Elisa Daldini,[†] Lucienne Juillerat-Jeanneret,[‡] and Paul J. Dyson[*,†]**

*Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland, and University Institute of Pathology, Centre Hospitalier Universitaire Vaudois (CHUV), CH-1011 Lausanne, Switzerland*

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In order to utilize macromolecules for drug targeting and delivery, a strategy to tether organometallic ruthenium–arene drugs to carrier protein molecules was developed. The approach involves the design of a drug fragment capable of conjugating to linker molecules on a modified carrier protein via hydrazone bond formation. The proof-of-concept using recombinant human serum albumin is described.

Drug targeting, i.e., the specific delivery of a drug to cancer cells, may be achieved by the use of targeting groups or by tuning the chemical and physical characteristics of the drug or drug carrier, such as hydrophobicity and molecular size.[1] One passive targeting method that has been widely utilized exploits the so-called “enhanced permeability and retention (EPR)” effect of macromolecules on tumors.[2] The EPR effect is based on the observation that macromolecules are able to penetrate the leaky vasculature surrounding the tumor. As a result of the increased permeability, the macromolecules “selectively” permeate the tumor tissues as compared to healthy tissues. In addition, their lymphatic drainage system is impaired, which results in an accumulation of the macromolecules at the tumor site. Different types of macromolecules have been used as carrier molecules, including liposomes, dendrimers, poly(ethylene glycol) polymers, nanoparticles, and protein biomolecules.[3]

In particular, human serum albumin (HSA) is known to accumulate in tumors, being taken up by tumor cells at increased levels compared to normal cells, and has been exploited as the carrier conjugate of various organic anticancer drugs such as chlorambucil, doxorubicin, and paclitaxel.[4] The main role of HSA is to maintain the osmotic pressure in the blood and to scavenge free radicals as an antioxidant. It is an attractive macromolecular carrier, given its nontoxicity and nonimmunogencity and that it is available in pure form. Chlorambucil and paclitaxel conjugated to HSA exhibit cytotoxicity comparable to that of the parent drugs in vitro but are less toxic in vivo.[5] In addition, a doxorubicin prodrug, which exploits endogenous serum albumin as a drug carrier, also showed a superior antitumor effect on murine renal cell carcinoma in vivo.[6] Recombinant HSA (rHSA) purified from yeast (*Pichia pastoris*) is also commercially available and has been tested in clinical trials, with no adverse effects reported.[4]

In recent years, there has been growing interest in studying ruthenium-based compounds as potential anticancer drug candidates, following the successful completion of two ruthenium(III) compounds in phase I clinical trials (see Figure 1).[7] In addition, organometallic ruthenium(II)−arene complexes bearing the 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]-decane ligand (known as RAPTA complexes) exhibit favorable pharmacological profiles in vitro and in vivo for application as antitumor compounds.[8,9] It is therefore worthwhile to develop a system to conjugate RAPTA moieties to a carrier protein molecule such as rHSA for passive drug targeting.

* To whom correspondence should be addressed. E-mail: paul.dyson@epfl.ch.
[†] Ecole Polytechnique Fédérale de Lausanne (EPFL).
[‡] Centre Hospitalier Universitaire Vaudois (CHUV).

(1) Yokoyama, M. *J. Artif. Organs* **2005**, *8*, 77−84.
(2) Modi, S.; Jain, J. P.; Domb, A. J.; Kumar, N. *Curr. Pharmaceut. Des.* **2006**, *12*, 4785−4796.
(3) Haag, R.; Kratz, F. *Angew. Chem., Int. Ed.* **2006**, *45*, 1198−1215.
(4) Chuang, V. T. G.; Kragh-Hansen, U.; Otagiri, M. *Pharm. Res.* **2002**, *19*, 569−577.
(5) (a) Dosio, F.; Brusa, P.; Crosasso, P.; Arpicco, S.; Cattel, L. *J. Controlled Release* **1997**, *47*, 293−304. (b) Kratz, F.; Beyer, U.; Roth, T.; Schutte, M. T.; Unold, A.; Fiebig, H. H.; Unger, C. *Arch. Pharm. (Weinheim)* **1998**, *331*, 47−53.
(6) (a) Kratz, F.; Muller-Driver, R.; Hofmann, I.; Drevs, J.; Unger, C. *J. Med. Chem.* **2000**, *43*, 1253−1256. (b) Kratz, F.; Warnecke, A.; Scheuermann, K.; Stockmar, C.; Schwab, J.; Lazar, P.; Druckes, P.; Esser, N.; Drevs, J.; Rognan, D.; Bissantz, C.; Hinderling, C.; Folkers, G.; Fichtner, I.; Unger, C. *J. Med. Chem.* **2002**, *45*, 5523−5533.
(7) (a) Hartinger, C. G.; Zorbas-Seifried, S.; Jakupec, M. A.; Kynast, B.; Zorbas, H.; Keppler, B. K. *J. Inorg. Biochem.* **2006**, *100*, 891−904. (b) Jakupec, M. A.; Arion, V. B.; Kapitza, S.; Reisner, E.; Eichinger, A.; Pongratz, M.; Marian, B.; Graf v. Keyserlingk, N.; Keppler, B. K. *Int. J. Clin. Pharmacol. Ther.* **2005**, *43*, 595−596. (c) Alessio, E.; Mestroni, G.; Bergamo, A.; Sava, G. *Curr. Top. Med. Chem.* **2004**, *4*, 1525−1535.
(8) (a) Ang, W. H.; Daldini, E.; Scolaro, C.; Scopelliti, R.; Juillerat-Jeannerat, L.; Dyson, P. J. *Inorg. Chem.* **2006**, *45*, 9006−9013. (b) Ang, W. H.; Dyson, P. J. *Eur. J. Inorg. Chem.* **2006**, 4003−4018. (c) Dyson, P. J.; Sava, G. *Dalton Trans.* **2006**, 1929−1933. (d) Gossens, C.; Dorcier, A.; Dyson, P. J.; Rothlisberger, U. *Organometallics* **2007**, *26*, 3969−3975.


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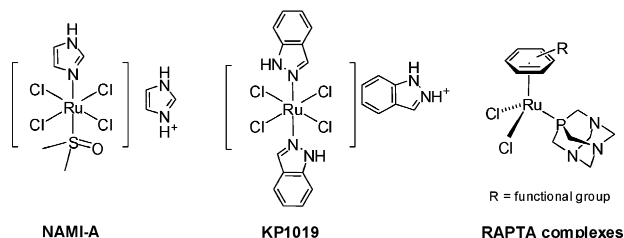



**Figure 1.** Ruthenium-based complexes investigated for anticancer activity.

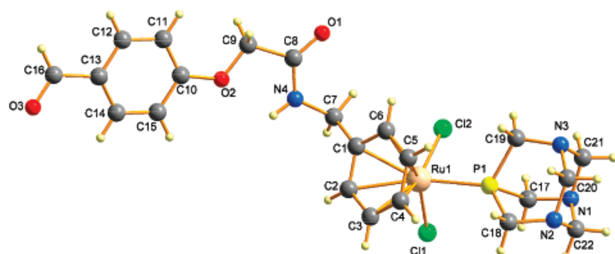
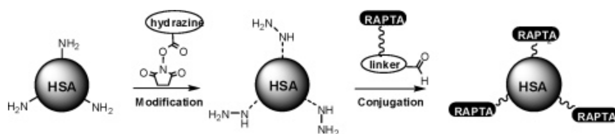


**Figure 2.** Ball-and-stick representation of **3**. Atoms are spheres of arbitrary diameter. The DMF solvent molecule is omitted for clarity. Key bond distances (Å) and angles (deg): Ru−$C_{av}$, 2.228; Ru−P, 2.308(4); Ru−$Cl_{av}$, 2.438(8); Cl−Ru−Cl, 86.61(14); P−Ru−$Cl_{av}$, 84.8(3).

**Scheme 1**




Ideally, the coordination sphere of the RAPTA moiety should remain as unperturbed as possible and, therefore, modification at the arene ring is preferred. In addition, because the reactive ruthenium center is susceptible to both nucleophilic and redox reagents, a mild conjugation method that connects the RAPTA moiety to the protein by means of a linker is required. An optimal conjugation method would be to cross-link RAPTA to biomolecules via acid-labile hydrazone bonds using aldehyde and hydrazine functional groups based on well-established methods. Indeed, the lability of the hydrazone bond under acidic conditions has been exploited for targeted release of drugs after cellular uptake.[5] We therefore decided to build a RAPTA fragment containing an aldehyde bond, which can conjugate to rHSA functionalized with hydrazine groups, by means of the hydrazone bond (see Scheme 1).

The ruthenium fragment was synthesized in two main parts via a reported procedure.[9] First, the arene ligand was prepared from 1-methylamine-1,4-cyclohexadiene (**1**) in three steps (see Scheme 2 and the Supporting Information). Second, the functionalized arene ligand was reacted with hydrated ruthenium(III) chloride in ethanol under reflux for 16 h to yield the arene-capped ruthenium(II) dimer **2**. The subsequent reaction with pta was carried out in degassed *N*,*N*-dimethylformamide (DMF) and monitored using $^{31}P\{^1H\}$ NMR

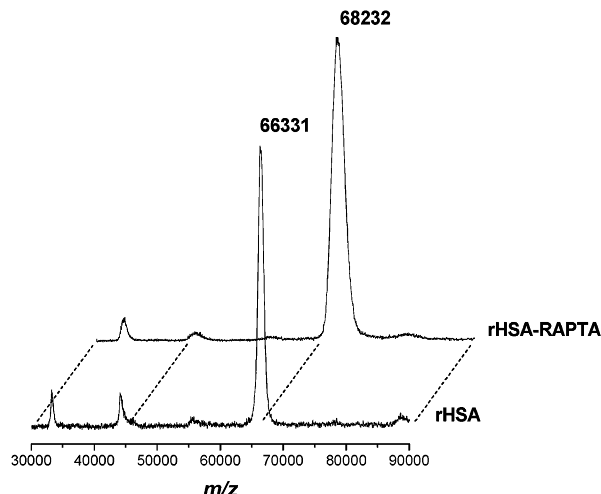
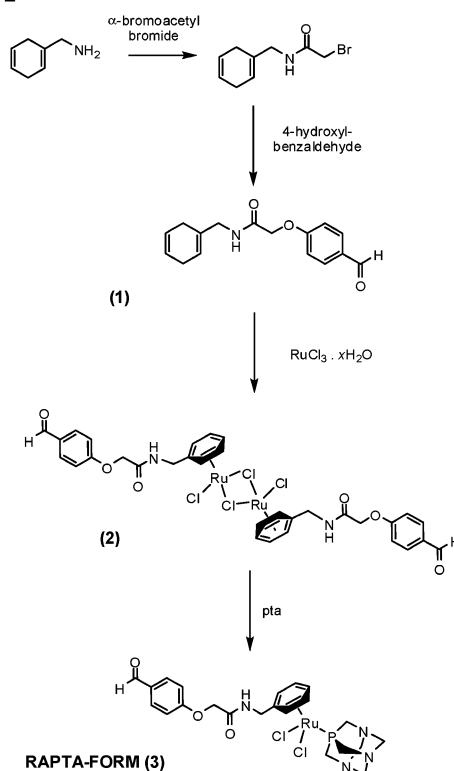



**Figure 3.** MALDI-TOF mass spectra of rHSA and rHSA−RAPTA conjugates.

**Scheme 2**




spectroscopy to yield the target complex RAPTA-FORM (**3**) in good yield. The product was characterized spectroscopically by negative-mode electrospray ionization mass spectrometry (nESI-MS) and $^1H$ and $^{31}P\{^1H\}$ NMR spectroscopy. In particular, its solid-state structure, determined using X-ray crystallography,[10] revealed that the bond parameters around the ruthenium(II) are remarkably similar to those of RAPTA-C, implying that the coordination sphere of the ruthenium(II) center is largely preserved (see Figure 2). The structure

(9) (a) Scolaro, C.; Bergamo, A.; Brescacin, L.; Delfino, R.; Cocchietto, M.; Laurenczy, G.; Geldbach, T. J.; Sava, G.; Dyson, P. J. *J. Med. Chem.* **2005**, *48*, 4161−4171. (b) Scolaro, C.; Geldbach, T. J.; Rochat, S.; Dorcier, A.; Gossens, C.; Bergamo, A.; Cocchietto, M.; Tavernelli, I.; Sava, G.; Rothlisberger, U.; Dyson, P. J. *Organometallics* **2006**, *25*, 756−765.

(10) Crystal data for **3**: $C_{22}H_{27}Cl_2N_4O_3PRu\cdot0.5DMF$, $M_w$ = 634.96, crystal system = monoclinic, $a$ = 20.93(2) Å, $b$ = 17.304(8) Å, $c$ = 6.992(3) Å, $\alpha = \gamma$ = 90°, $\beta$ = 90.20(6)°, $V$ = 2532(3) Å$^3$, $T$ = 100(2) K, space group = $P2_1/c$, $Z$ = 4, $\lambda$(Mo K) = 0.710 73 Å, 25 582 reflections collected, 4012 independent reflections, $R_{int}$ = 0.1341, R1 [$I > 2(I)$] = 0.0886, wR2 (all data) = 0.2299, GOF = 1.122.

**Table 1.** Inhibition of Cancer Cell Growth ($IC_{50}$) for Test Compounds and rHSA Conjugates after 72 h

| | A2780 ovarian carcinoma |
|---|---|
| rHSA | >75 $\mu$M[a] |
| rHSA−hydrazine | >75 $\mu$M[a] |
| rHSA−RAPTA | 11 $\mu$M |
| RAPTA-FORM **3** | 288 $\mu$M |
| RAPTA-C | >300 $\mu$M |

[a] Maximum concentration possible.

of **1** was also confirmed by X-ray crystallography,[11] and the bond parameters of the functional part of the molecule are very similar to those of the coordinated system (see the Supporting Information).

The functionalization of the rHSA protein was carried using established protocols (see the Supporting Information for full details). Briefly, the protein was modified with the succinyl HCl terephthalic hydrazine linker, which reacts with amine groups on the lysine residues of the protein. Because excess modification of the hydrophobic linkers can result in the precipitation of the protein, the optimal reaction conditions were determined empirically to be within 5−10-fold stoichiometric excess of the linker molecule. Upon modification, the protein is purified and conjugated with **3** in phosphate-buffered saline (pH 7.4). The conjugated protein is further purified and analyzed using UV−vis absorption spectroscopy and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The presence of the conjugated hydrazone bond is indicated by an increase in absorbance at 317 nm, which is absent in rHSA, hydrazine-modified rHSA, and **3**. The MALDI-TOF mass spectra obtained indicated an increase of approximately 1900 Da, equivalent to the presence of three to four RAPTA moieties. The mass spectra also showed that the parent rHSA molecule was completely consumed during the conjugation (see Figure 3). However, the broadening of the mass peak suggests that conjugation was not completely homogeneous and that the final protein solution probably contained a mixture of rHSA species with different numbers of RAPTA conjugates.

Using the A2780 ovarian carcinoma cell line, rHSA, rHSA modified with the hydrazine linker, and rHSA conjugated with RAPTA were tested for their ability to inhibit cancer cell growth in vitro. The protein concentration was determined using the Bradford assay, and the cells were tested to a maximum protein concentration of 5 mg/mL, equivalent to 75 $\mu$M. Whereas rHSA and the hydrazine-modified derivative did not significantly affect the cell growth within the concentration range tested, a positive response was observed in the A2780 cell line exposed to rHSA conjugated with RAPTA. The $IC_{50}$ value is 20-fold lower than that of **3**, even if one considers that four RAPTA moieties are present (Table 1). It is worth noting that RAPTA-C, the prototype compound with a *p*-cymene ring, is also nontoxic toward A2780 cells with $IC_{50} > 300\ \mu$M, further indicating the remarkable effect provided by the conjugation of the ruthenium(II)−arene unit to rHSA.

In comparison, paclitaxel drug moieties conjugated to HSA via ester bonds (6 or 30 molecules per HSA) were less active than the drug in free form.[5] This was attributed to the labile ester bonds, which may be hydrolyzed before cellular uptake. Such a hypothesis is supported by the observation that while the HSA−chlorambucil conjugate with ester bond linkers was not active against cancer cells, a similar derivative with acetaldehyde carboxylic hydrazone bond linkers was active. As mentioned above, such hydrazone bonds have been shown to cleave under acidic conditions, thus providing a means for the targeted release of the drug in the acidic environment of lysosomes within the tumor cell. This phenomenon could explain the improved efficacy in the rHSA−RAPTA conjugate reported herein. Because HSA in known to be taken up by cells via endocytosis, the facilitated uptake of the drug conjugate coupled with the controlled release of the RAPTA drug moiety could correspond to the key reason for the improvement in drug efficacy. Clearly, these potential mechanisms would need to be investigated further in order to develop other organometallic drug−protein conjugates with optimized activities against cancer cells.

In conclusion, a strategy to tether an organometallic ruthenium(II)−arene anticancer compound to rHSA was developed with a view to develop a drug that could selectively accumulate in tumor cells. Preliminary results indicate that the strategy is viable and that rHSA could be exploited as a carrier biomolecule for drug delivery of RAPTA complexes in vivo, with the prospect of adjusting the loading capacity for tailored effects.

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**Supporting Information Available:** Crystallographic data of **1** and **3** in CIF format, synthesis of **3**, and protocol for the inhibition of cell growth experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Crystal data for **1**: $C_{16}H_{17}NO_3$, $M_w$ = 271.31, crystal system = monoclinic, $a$ = 5.2366(5) Å, $b$ = 12.6144(19) Å, $c$ = 21.364(2) Å, $\alpha = \gamma = 90°$, $\beta$ = 96.086(8)°, $V$ = 1403.3(3) Å$^3$, $T$ = 100(2) K, space group = $P2_1/c$, $Z$ = 4, $\lambda$(Mo K) = 0.710 73 Å, 25 814 reflections collected, 3211 independent reflections, $R_{int}$ = 0.0591, R1 [$I > 2(I)$] = 0.0511, wR2 (all data) = 0.1018, GOF = 1.128.