MedChemComm

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/medchemcomm

Journal Name

RSCPublishing

COMMUNICATION

Cytotoxic Peptide Conjugates of Dinuclear Arene Ruthenium Trithiolato Complexes†

Cite this: DOI: 10.1039/x0xx00000x

Federico Giannini[a,l], Marco Bartoloni[a,l], Lydia E. H. Paul[a], Georg Süss-Fink[b] Jean-Louis Reymond[a,*] and Julien Furrer[a,*]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

In order to improve the water-solubility of dinuclear thiolato-bridged arene ruthenium complexes, a new series was synthesized by conjugation to octaarginine, octalysine, and *cyclo*[Lys-Arg-Gly-Asp-D-Phe] using the chloroacetyl thioether (ClAc) ligation, resulting in cytotoxic conjugates against A2780 human ovarian cancer cells (IC_{50} = 2 - 8 μM) and against the cisplatin resistant line A2780cisR (IC_{50} = 7 - 15 μM). These metal complexes represent, to the best of our knowledge, the most cytotoxic ruthenium bioconjugates reported so far.

Introduction

The success of *cis*-$[Pt(NH_3)_2Cl_2]$ (cisplatin) and its analogues as the most effective chemotherapeutic agents in clinical use since over 30 years[1] has led to further research into transition metal complexes as chemotherapeutic agents.[2, 3] Ruthenium complexes represent an attractive alternative to platinum because they are generally non-toxic yet sometimes show selective toxicity for cancer cells.[4-6] For example the Ru(III) complexes KP1019, most likely binding DNA[6, 7] and NAMI-A, whose mechanism of action is still not fully understood,[8] are currently in clinical trials for the treatment of metastatic non-small cell lung cancer (NAMI-A)[8] and colorectal, endometrial, melanoma and bladder carcinomas (KP1019).[9] One limitation of transition metal-based drugs is their relatively modest potency (IC_{50} > 3 μM for in vitro cytotoxicity in most cases) and limited water solubility, which requires high quantities of drug administration leading to undesirable side effects. Recently we showed that arene ruthenium complexes $[(\eta^6\text{-}p\text{-cymene})_2Ru_2(\mu_2\text{-}SR)_3]^+$ and $[(\eta^6\text{-}p\text{-cymene})_2Ru_2(\mu_2\text{-}SR^1)(\mu_2\text{-}SR^2)_2]^+$ are among the most cytotoxic ruthenium complexes so far, with submicromolar IC_{50} values (IC_{50} up to 0.03 μM) against A2780 human ovarian cancer cells and their cisplatin-resistant mutant variant A2780cisR.[10-13]

Despite of their unusually high potency, our Ru complexes were not selective for cancer cells[14] and showed very low water-solubility, severely limiting perspectives for in vivo studies. To address this limitation, we set out to investigate their conjugation to cell-penetrating peptides (CPPs), which might also improve targeting.[15, 16] This strategy has been used previously with other cytotoxic metal complexes such as platinum,[17] osmium,[18] rhodium(III)[19] and ruthenium(II) complexes.[20-22] Ruthenium compounds $[(\eta^6\text{-}p\text{-cymene})_2Ru_2(S\text{-}C_6H_4\text{-}p\text{-}SH)(S\text{-}CH_2\text{-}C_6H_4\text{-}^tBu)_2]Cl$ (**1**) and $[(\eta^6\text{-}p\text{-cymene})_2Ru_2(S\text{-}C_6H_4\text{-}p\text{-}SH)(S\text{-}CH_2\text{-}CH_2\text{-}C_6H_5)_2]Cl$ (**2**), carrying a free thiol group, were conjugated to octaarginine (R_8) and octalysine (K_8) both known to show comparable cellular uptake,[23] and to *cyclo*[Lys(ClAc)-Arg-Gly-Asp-D-Phe] a ligand of the $\alpha_v\beta_3$ integrin receptor which is selectively overexpressed near tumours.[24] Conjugation was accomplished by a chemoselective chloroacetyl thioether (ClAc) ligation,[25] a method which provides excellent yields with respect to the metal complex in contrast to previously reported peptide coupling approaches requiring excess of the metal complex. The resulting peptide conjugates **1-R8**, **1-K8**, **1-RGD**, **2-R8**, **2-K8** and **2-RGD** showed good water-solubility and, most remarkably, a largely preserved cytotoxicity of the parent complexes (IC_{50} ~ 2-8 μM) together with a significant gain in selectivity towards cancer cells. To the best of our knowledge these new Ru-peptide conjugates represent the most cytotoxic ruthenium bioconjugates to date.

Results and Discussion

Design and Synthesis

Cytotoxic transition metal complexes have been conjugated to peptides by amide bond formation between a carboxyl-functionalized metal complex and the free N-terminus of the peptide as the last step of a solid-phase peptide synthesis (SPPS), which required 3-5 equivalents of the metal complex.[17, 18, 26, 27] We envisioned a more efficient conjugation by chemical ligation between the metal complex and a purified peptide. Considering the sulphur-rich nature of our arene-ruthenium complexes, a chloroacetyl cysteine thioether (ClAc) ligation of a thiolated metal complex to an N-terminal chloroacetyl group was investigated.[25, 28] Thiolated metal complexes (**1**) and (**2**) were obtained by reaction of the *p*-cymene-ruthenium dichloride dimer $[(\eta^6\text{-}p\text{-cymene})RuCl(\mu_2\text{-}Cl)]_2$ with two equivalents of

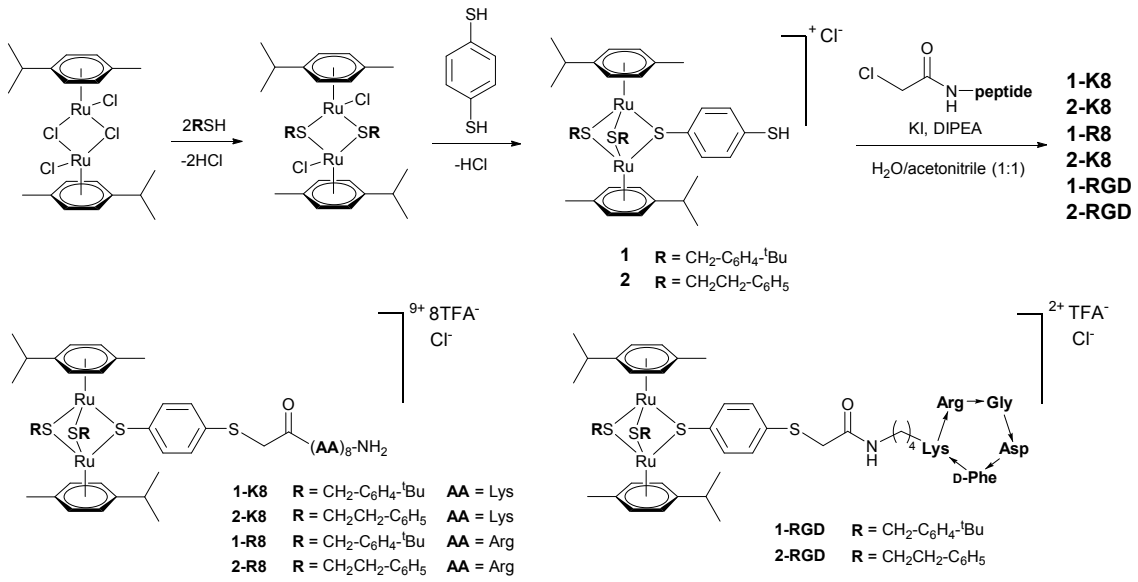


Figure 1. Synthesis of peptide-complex conjugates **1-K8/R8/RGD** and **2-K8/R8/RGD**.

(4-tert-butylphenyl)methanethiol or 2-phenylethanethiol as previously reported,[29] followed by reaction of these intermediates with 6 equivalents of 1,4-dithiophenol in refluxing ethanol during 16 h. Note that the same reaction with 4-mercaptobenzoic acid did not yield the expected product, precluding an amide bond strategy for peptide conjugation.

Complexes **1** and **2** were conjugated to N-chloroacetylated octaarginine and octalysine and *cyclo*[Lys(ClAc)-Arg-Gly-Asp-D-Phe] by ClAc ligation in water/acetonitrile (1:1) mixtures (6 mM Ru-complex) with a slight excess of N-chloroacetylated peptide in presence of potassium iodide and diisopropylamine as base. LC-MS monitoring revealed that the ligations ran to completion within 10-15 min to form the expected conjugates, which were purified by preparative RP-HPLC (Table 1). Isolated yields spanned between 33% (**1-K8**) and quantitative (**2-RGD**) with respect to the metal complex. Conjugates **1-K8**, **2-K8**, **1-R8** and **2-R8**, carrying nine positive charges, were completely water-soluble, whereas dicationic conjugates **1-RGD** and **2-RGD**, although non soluble in pure water, were considerably more polar than the corresponding ruthenium complexes, as revealed by RP-HPLC analysis. All conjugates were characterized by ^{1}H-NMR, ESI-mass spectrometry and elemental analysis (see Figures in the Supplementary Information).

Table 1. Quantity obtained, yields of the thioether ligation reactions and ESI-MS data for the six conjugates.

complex	quantity (mg)	yield (%)	$[M+xH]^{(x+1)+}$ calc./obs.
1-K8	18.2	33	1026.99/1026.99 (x = 1)
2-K8	34.5	64	984.94/984.95 (x = 1)
1-R8	20.2	34	759.68/759.68 (x = 2)
2-R8	40.1	69	731.65/731.65 (x = 2)
1-RGD	11.3	69	1614.50/1614.50 (x = 0)
2-RGD	15.3	99	1530.40/1530.41 (x = 0)

Cytotoxicity

Cytotoxicity was evaluated in A2780 human ovarian cancer cells and its cisplatin resistant line A2780cisR as well as in HEK293 cells as a model of non-cancerous cells. Complexes **1** and **2** showed submicromolar activity towards A2780 cells, which is weaker than our previous ruthenium complexes,[11-13] yet these values are still among the best ones for cytotoxic transition metal complexes.

The peptide conjugated Ru-complexes showed IC_{50} values in the range 2 - 6 μM against A2780 cells and in the range 7 - 15 μM against A2780cisR cells, corresponding to a reduction of approximately 4-fold (A2780) and 5-8 fold (A2780cisR) compared to the unconjugated complexes **1** and **2** (Table 2). Interestingly, the IC_{50} of the peptide conjugates against HEK293 cells were somewhat higher (3.5 - 8 μM) compared to the IC_{50} against A2780 cells, resulting in a slight selectivity of the conjugates towards these cancer cells. On the other hand, in contrast to most of our previous ruthenium complexes, for which the IC_{50} against A2780cisR cells were similar or even lower than the IC_{50} against A2780 cells,[11-13] the IC_{50} of the peptide conjugates against A2780cisR cells were also higher (7.2 – 13.3 μM) compared to the IC_{50} against A2780 cells. Generally, the conjugates are found to be approximately equally active than cisplatin against the parent cell line A2780, and about three times more active against the cisplatin-resistant cell line A2780cisR. Whereas the resistance factor for cisplatin as applied to the cell lines A2780 and A2780cisR is 7.6, the corresponding values for the conjugates ranged from 1.2 to 6.7. In line with our previous ruthenium complexes, the results suggest that the conjugates have been able to partly overcome resistance in A2780cisR cell lines.

Overall, peptide conjugation resulted in a very strong increase in water solubility at the expense of a significant yet acceptable reduction in activity and a slight gain in selectivity. Despite their weaker activity compared to **1** and **2**, the peptide conjugates still represent the most active transition-metal peptide conjugates reported to date, with an activity comparable to that of cisplatin (IC_{50} = 2.9 μM).

Table 2. IC_{50} (μM) values selectivity and resistance factor for **1-2** and their peptide conjugates.

Complex	IC_{50} (A2780)	IC_{50} (A2780cisR)	IC_{50} (HEK293)	Selectivity[+]	Resistance Factor[l]
1	$0.5^{\pm 0.01}$	$0.7^{\pm 0.1}$	$0.6^{\pm 0.06}$	1.2	1.4
2	$0.6^{\pm 0.02}$	$1.4^{\pm 0.3}$	$0.4^{\pm 0.02}$	0.7	2.3
1-K8	$2.5^{\pm 0.5}$	$7.3^{\pm 1.1}$	$7.7^{\pm 0.7}$	3.1	2.9
2-K8	$2.0^{\pm 0.6}$	$13.3^{\pm 4.3}$	$3.5^{\pm 0.4}$	1.8	6.7
1-R8	$4.2^{\pm 0.3}$	$8.1^{\pm 0.8}$	$8.0^{\pm 0.8}$	1.9	1.9
2-R8	$6.0^{\pm 0.4}$	$7.2^{\pm 1.6}$	$6.0^{\pm 0.7}$	1.0	1.2
1-RGD	$4.1^{\pm 0.1}$	$7.3^{\pm 0.7}$	$7.7^{\pm 1}$	1.9	1.8
2-RGD	$4.8^{\pm 0.5}$	$15.5^{\pm 6}$	$4.5^{\pm 0.80}$	0.9	3.2
cisplatin	$2.9^{\pm 0.2*}$	$21.9^{\pm 0.8*}$	$88.7^{\pm 23}$	30.1	7.6

*Value taken from ref 13 [+]Selectivity = IC_{50} (HEK293) / IC_{50}(A2780)
[l]Resistance Factor = IC_{50} (A2780cisR) / IC_{50}(A2780)

Considering the well-known cell-penetrating properties of R8 and K8, the reduced activity of the conjugates compared to **1** and **2** probably reflects a combination of a solubilisation effect reducing the tendency to stick to the cells, compensated by a cell penetrating effect increasing cellular delivery. Nevertheless the cytotoxicity results imply that a cell-penetrating effect might not be necessary, which might explain the activity of the RGD conjugate. Note that although the cell penetrating effect of R8 and K8 is well documented in the case of fluorescein conjugates,[20] cellular localisation is strongly dependent on the exact nature of the conjugates, precluding a direct investigation of the localization of the complexes since these are not fluorescent.

Conclusions

In this work, new dinuclear arene ruthenium trithiolato conjugates were synthesized by an efficient thioether ligation between the free thiol group of the water insoluble Ru-complexes **1** or **2** and N-chloroacetylated peptides to produce conjugates which were soluble and stable in water. Although less cytotoxic compared to the parent compounds, following the tendency observed with other ruthenium and osmium conjugates, conjugates **1-K8/R8/RGD** and **2-K8/R8/RGD** represent, to the best of our knowledge, the most cytotoxic ruthenium bioconjugates reported so far.

The activity of our conjugates is noteworthy because conjugation often results in loss of activity.[18, 26] For instance, the osmium complex [(η^6-biphenyl)Os(picolinate)Cl] was cytotoxic against A2780 cells (IC_{50} = 4.5 μM), but its toxicity dropped to IC_{50} = 72 μM for its R5-conjugate and 33 μM for its R8 conjugate.[18] A complete quenching of activity was reported for some manganese complex-peptide conjugates.[30] Reports that peptide conjugation may also increase the activity of the metal complexes, as shown for platinum(IV) analogues of oxaliplatin conjugated to a TAT-peptide fragment (IC_{50} = 55 → 1.4 μM),[17] and Ru-(arene) complex conjugated to the neuropeptide [Leu5]-encephalin (IC_{50} = no activity → 13 μM),[27] suggests that further engineering of the peptide sequence, possibly using a selectively cleavable linker, might lead to more potent and selective Ru-peptide conjugates.

Acknowledgments

This work was funded by the Swiss National Science Foundation, project 200021-144421 (JF)

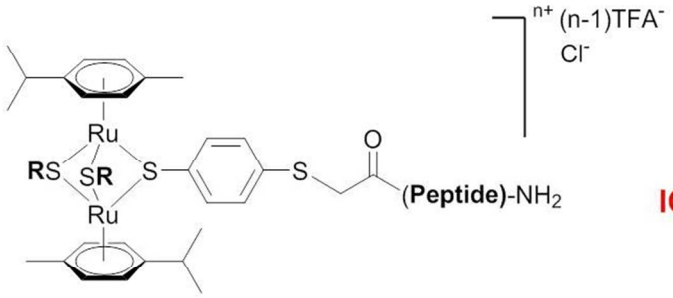
Notes and references

[a]Departement für Chemie und Biochemie, Universität Bern, CH-3012 Bern, Switzerland.
jean-louis.reymond@dcb.unibe.ch
julien.furrer@dcb.unibe.ch
[b]Institut de Chimie, Université de Neuchâtel, CH-2000 Neuchâtel, Switzerland.
[l]both authors contributed equally to this work

† Electronic Supplementary Information (ESI) available: Experimental procedure, spectroscopic data]. See DOI: 10.1039/c000000x/

1. N. J. Wheate, S. Walker, G. E. Craig and R. Oun, *Dalton Trans.*, 2010, **39**, 8113-8127.
2. P. C. A. Bruijnincx and P. J. Sadler, *Curr. Opin. Chem. Biol.*, 2008, **12**, 197-206.
3. S. Komeda and A. Casini, *Curr. Top. Med. Chem.*, 2012, **12**, 219-235.
4. G. Süss-Fink, *Dalton Trans.*, 2010, **39**, 1673-1688.
5. W. H. Ang, A. Casini, G. Sava and P. J. Dyson, *J. Organomet. Chem.*, 2011, **696**, 989-998.
6. A. Bergamo, C. Gaiddon, J. H. M. Schellens, J. H. Beijnen and G. Sava, *J. Inorg. Biochem.*, 2012, **106**, 90-99.
7. C. G. Hartinger, S. Zorbas-Seifried, M. A. Jakupec, B. Kynast, H. Zorbas and B. K. Keppler, *J. Inorg. Biochem.*, 2006, **100**, 891-904.
8. J. M. Rademaker-Lakhai, D. van den Bongard, D. Pluim, J. H. Beijnen and J. H. M. Schellens, *Clin. Cancer Res.*, 2004, **10**, 3717-3727.
9. C. G. Hartinger, M. A. Jakupec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P. J. Dyson and B. K. Keppler, *Chem. Biodiversity*, 2008, **5**, 2140-2155.
10. F. Giannini, G. Süss-Fink and J. Furrer, *Inorg. Chem.*, 2011, **50**, 10552-10554.
11. F. Giannini, J. Furrer, A.-F. Ibao, G. Süss-Fink, B. Therrien, O. Zava, M. Baquie, P. J. Dyson and P. Stepnicka, *J. Biol. Inorg. Chem.*, 2012, **17**, 951-960.
12. F. Giannini, J. Furrer, G. Süss-Fink, C. M. Clavel and P. J. Dyson, *J. Organomet. Chem.*, 2013, **744**, 41-48.
13. F. Giannini, L. E. H. Paul, J. Furrer, B. Therrien and G. Süss-Fink, *New J. Chem.*, 2013, **37**, 3503-3511.
14. F. Giannini, J. Furrer, G. Süss-Fink, C. M. Clavel and P. J. Dyson, *unpublished results*.
15. K. M. Stewart, K. L. Horton and S. O. Kelley, *Org. Biomol. Chem.*, 2008, **6**, 2242-2255.
16. P. A. Wender, W. C. Galliher, E. A. Goun, L. R. Jones and T. H. Pillow, *Adv. Drug Delivery Rev.*, 2008, **60**, 452-472.
17. S. Abramkin, S. M. Valiahdi, M. A. Jakupec, M. Galanski, N. Metzler-Nolte and B. K. Keppler, *Dalton Trans.*, 2012, **41**, 3001-3005.
18. S. H. van Rijt, H. Kostrhunova, V. Brabec and P. J. Sadler, *Bioconjugate Chem.*, 2011, **22**, 218-226.
19. J. Brunner and J. K. Barton, *Biochemistry*, 2006, **45**, 12295-12302.

20. C. A. Puckett and J. K. Barton, *J. Am. Chem. Soc.*, 2009, **131**, 8738-8739.
21. C. A. Puckett and J. K. Barton, *Bioorg. Med. Chem.*, 2010, **18**, 3564-3569.
22. L. Cosgrave, M. Devocelle, R. J. Forster and T. E. Keyes, *Chem. Commun.*, 2010, **46**, 103-105.
23. A. El-Sayed, I. A. Khalil, K. Kogure, S. Futaki and H. Harashima, *J. Biol. Chem.*, 2008, **283**, 23450-23461.
24. A. Meyer, J. Auemheimer, A. Modlinger and H. Kessler, *Curr. Pharm. Des.*, 2006, **12**, 2723-2747.
25. N. A. Uhlich, T. Darbre and J.-L. Reymond, *Org. Biomol. Chem.*, 2011, **9**, 7071-7084.
26. F. Barragán, D. Carrion-Salip, I. Gómez-Pinto, A. González-Cantó, P. J. Sadler, R. de Llorens, V. Moreno, C. González, A. Massaguer and V. Marchán, *Bioconjugate Chem.*, 2012, **23**, 1838-1855.
27. S. M. Meier, M. Novak, W. Kandioller, M. A. Jakupec, V. B. Arion, N. Metzler-Nolte, B. K. Keppler and C. G. Hartinger, *Chem.--Eur. J.*, 2013, **19**, 9297-9307.
28. W. Lindner and F. A. Robey, *Int. J. Pept. Protein Res.*, 1987, **30**, 794-800.
29. A. F. Ibao, M. Gras, B. Therrien, G. Suss-Fink, O. Zava and P. J. Dyson, *Eur. J. Inorg. Chem.*, 2012, 1531-1535.
30. K. Splith, I. Neundorf, W. Hu, H. W. P. N'Dongo, V. Vasylyeva, K. Merz and U. Schatzschneider, *Dalton Trans.*, 2010, **39**, 2536-2545.



IC$_{50}$ (A2780): 2 - 6 μM

R = CH_2-C_6H_4-^{t}Bu or CH_2CH_2-C_6H_5

Peptide = K_8, R_8 or *cyclo*[KRGDf]

254x190mm (96 x 96 DPI)