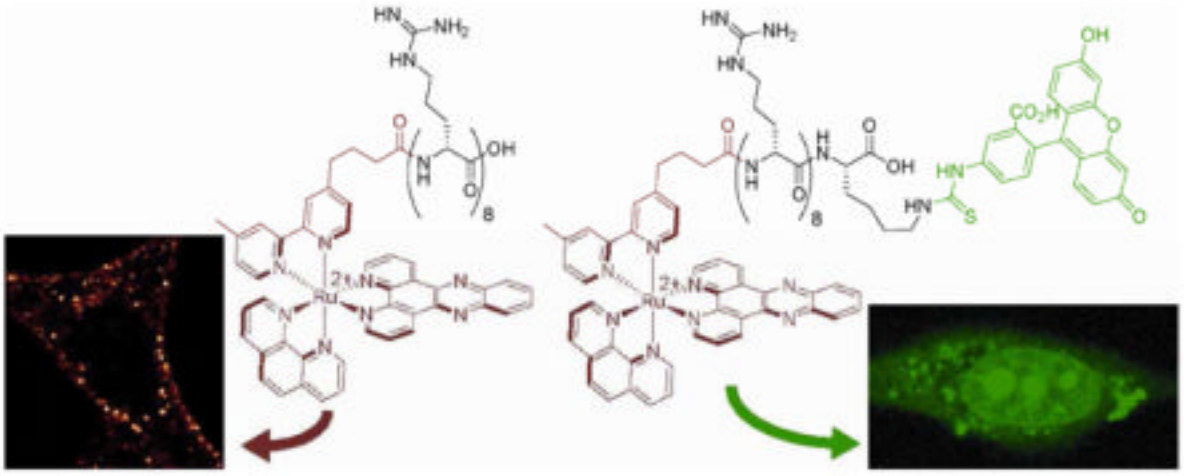
Published in final edited form as:
J Am Chem Soc. 2009 July 1; 131(25): 8738–8739. doi:10.1021/ja9025165.

Fluorescein Redirects a Ruthenium-Octaarginine Conjugate to the Nucleus

Cindy A. Puckett and **Jacqueline K. Barton***
Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA

Abstract



The cellular uptake and localization of a Ru-octaarginine conjugate with and without an appended fluorescein are compared. The inherent luminescence of the Ru(II) dipyridophenazine complex allows observation of its uptake without the addition of a fluorophore. Ru-octaarginine-fluorescein stains the cytosol, nuclei and nucleoli of HeLa cells under conditions where the Ru-octaarginine conjugate without fluorescein shows only punctate cytoplasmic labeling. At higher concentrations, however, Ru-octaarginine without the fluorescein tag does exhibit cytoplasmic, nuclear, and nucleolar staining. Attaching fluorescein to Ru-octaarginine lowers the threshold concentration required for diffuse cytoplasmic labeling and nuclear entry. Hence, the localization of the fluorophore-bound peptide cannot serve as a proxy for that of the free peptide.

The cellular uptake and localization characteristics of molecular probes and therapeutics are fundamental to their efficacy. The rational construction of molecules with the desired uptake kinetics and subcellular localization is however not straightforward. Cell-penetrating peptides (CPPs), such as the HIV Tat peptide and oligoarginine, promote the cellular uptake of many cargos, including peptides, proteins, oligonucleotides, plasmids, and peptide nucleic acids.[1, 2] To evaluate their entry characteristics, organic fluorophores are routinely used as the CPP cargo. Some labs have varied the fluorophore to assess uptake and found some fluorophore-dependent changes.[3–6] However, uptake of the fluorophore-labeled peptide may not correlate

jkbarton@caltech.edu.

Supporting Information Available. Spectral confocal imaging of Ru-D-R8-fluor. This material is available free of charge via the Internet at http://pubs.acs.org.

with that of the free CPP. Here, using a luminescent Ru-octaarginine conjugate, we explore directly the effects of an attached organic fluorophore on its cellular uptake and distribution.

Many laboratories are focused on the design of novel metal complexes as cellular probes and for therapeutic application.[7] In our laboratory, 5,6-chrysenequinone diimine (chrysi) complexes of Rh(III) are found to selectively inhibit cellular proliferation in mismatch repair-deficient cells and these complexes are being explored as potential chemotherapeutic agents.[8] In an effort to target metal complexes to the nucleus more effectively, we previously prepared a chrysi complex of Rh(III) covalently tethered to D-octaarginine (D-R8) fluorescein and found that it rapidly localizes to the nucleus of HeLa cells.[9,10] As the Rh complex itself is not fluorescent, fluorescein was attached to monitor the subcellular distribution of this Rh-D-R8 conjugate.

We have also carried out studies using dipyridophenazine (dppz) complexes of Ru(II), which are luminescent, to examine cellular uptake characteristics of the octahedral transition metal complexes.[11] These dppz complexes serve as light switches for non-aqueous environments, since hydrogen bonding with water efficiently quenches the Ru luminescence.[12,13] As a result, in aqueous solution, the dppz complexes luminesce brightly only when bound to DNA or otherwise protected from water through binding to folded RNAs, or association with membranes or other macromolecular structures. Dppz complexes of Ru(II) are able to enter live cells, with uptake by passive diffusion enhanced by more lipophilic ligands.[11] Tris (polypyridyl) complexes of Ru(II) are furthermore stable to ligand dissociation and substitution. Thus, as decomposition or loss of ligands would render the Ru complex non-luminescent, the characteristic luminescence indicates that it remains intact in the cellular environment.

Here we compare directly cellular uptake by peptide conjugates of Ru(II) dppz with and without a fluorescent tag. Three Ru(II) dppz conjugates were synthesized: Ru-octaarginine (Ru-D-R8), Ru-octaarginine-fluorescein (Ru-D-R8-fluor), and Ru-fluorescein (Ru-fluor) (Figure 1). Ru(phen)(bpy′)(dppz)$^{2+}$ was coupled to the peptide in an analogous manner to that previously described (where bpy′ = 4-(3-carboxypropyl)-4′-methyl-2,2′-bipyridine).[9,14,15] The complexes were incubated with HeLa cells in complete medium (α-MEM with 10% FBS) at 37 °C under the following conditions: Ru-D-R8 at 2–20 μM for 30 min, Ru-D-R8-fluor at 2–5 μM for 30 min, and Ru-fluor at 5 μM for 30 min and 20 μM for 41 h. The samples were then rinsed with Hanks' balanced salt solution (HBSS) and imaged without fixation.[16]

HeLa cells incubated with Ru-D-R8 at 5 μM for 30 min exhibit punctate luminescence in the cytoplasm, with complete exclusion from the nucleus (Figure 1, top). The punctate distribution implicates endocytosis, a proposed internalization mechanism for oligoarginine CPPs, as its route into the cell.[17] Entrapment in endosomes would explain the lack of nuclear entry. In this context, the peptide changes the mode of uptake relative to unconjugated complexes, such as Ru(phen)(bpy′)(dppz)$^{2+}$, Ru(phen)$_2$dppz^{2+} and Ru(bpy)$_2$dppz^{2+}, which enter by passive diffusion and do not exhibit punctate staining.[11] As expected, for the peptide conjugates, cellular uptake is strongly enhanced compared to these unconjugated complexes; higher luminescence is evident in cell samples even after short incubation times. Notably, increasing the incubation time to 24 h does not change the subcellular localization of 5 μM Ru-D-R8.

Remarkably, the Ru-octaarginine conjugate containing an appended fluorescein (Ru-D-R8-fluor) enters the nucleus under the same incubation conditions for which the complex without fluorescein is excluded. Ru-D-R8-fluor shows diffuse cytoplasmic and nuclear fluorescence, strong nucleolar staining, and some punctate cytoplasmic staining when incubated at 5 μM for 30 min with HeLa (Figure 1, center). Some cells have numerous fluorescent punctate structures, while others have relatively few. The intensity of fluorescence in the nucleoli is roughly equal

to that of these punctate, vesicular structures. Nucleolar labeling is typical of D-octaarginine, as seen here, although not of L-octaarginine.[18] Notably, at this concentration, D-R8-fluor and the Rh(III) conjugate of D-R8-fluor also localize to the nucleus.[9]

Not surprisingly, the Ru-fluorescein conjugate lacking octaarginine is unable to enter the cell under the same incubation conditions for which its octaarginine counterparts can translocate (5 μM for 30 min). The complex is poorly internalized even following a longer incubation time with higher concentration (20 μM for 41 h) (Figure 1, bottom). Given its significantly lower positive charge, the complex cannot as effectively use the membrane potential as a driving force for cellular entry.[19]

At higher concentrations, the localization of Ru-D-R8 changes significantly. Up to 10 μM, the complex is restricted to punctate structures in the cytoplasm. At 15–20 μM, the cell population is heterogeneous. Some cells have only punctate cytoplasmic staining, while others exhibit additional diffuse cytoplasmic as well as nuclear and nucleolar staining (Figure 2). The fraction of cells in the latter population increases with concentration (Table 1).[20] As for the Ru-D-R8-fluor, the nucleolar and punctate staining are of similar intensity, with fainter nuclear and cytoplasmic staining. A concentration threshold for diffuse cytoplasmic and nuclear labeling is a feature of oligoarginine-fluorophore conjugates and has been reported previously.[18,20–21] Above the extracellular threshold concentration, the peptides are postulated to enter by a non-endocytic mechanism in addition to the endocytic mechanisms evident at lower concentrations. Markedly, the threshold for Ru-D-R8-fluor nuclear entry is between 2 and 5 μM, significantly lower than that for Ru-D-R8.

What role is fluorescein playing in this uptake? Fluorescein, due to its greater lipophilicity versus the Ru moiety, increases the interaction of Ru-D-R8-fluor with the cell membrane compared to Ru-D-R8. This high concentration at the cell surface could facilitate the non-endocytic uptake mechanism, promoting access to the cytosol, and ultimately, the nucleus, while low concentrations at the cell surface should limit the uptake to endocytosis, with consequent endosomal trapping, observed as punctate cytoplasmic staining.

Thus, as revealed by the luminescent ruthenium cargo, fluorophore tagging of a cell-penetrating peptide does more than supply luminescence. The molecular nature of the organic fluorophore affects the transport pathway and its subcellular localization. Hence, the localization of the fluorophore-bound peptide cannot simply serve as a proxy for that of the free peptide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the NIH (GM33309) and to the Caltech Biological Imaging Center.

References

1. (a) Stewart KM, Horton KL, Kelley SO. Org Biomol Chem 2008;6:2242–2255. [PubMed: 18563254] (b) Fischer R, Fotin-Mleczek M, Hufnagel H, Brock R. ChemBioChem 2005;6:2126–2142. [PubMed: 16254940]
2. Goun EA, Pillow TH, Jones LR, Rothbard JB, Wender PA. ChemBioChem 2006;7:1497–1515. [PubMed: 16972294]
3. Fischer R, Waizenegger T, Köhler K, Brock R. Biochim Biophys Acta 2002;1564:365–374. [PubMed: 12175919]

4. El-Andaloussi S, Järver P, Johansson HJ, Langel Ü. Biochem J 2007;407:285–292. [PubMed: 17627607]
5. Szeto HH, Schiller PW, Zhao K, Luo G. FASEB J 2005;19:118. [PubMed: 15489247]
6. The uptake characteristics of pyrrole-imidazole polyamides with different fluorophores have been extensively characterized and shown to vary with the nature of the fluorophore. See: (a)Best TP, Edelson BS, Nickols NG, Dervan PB. Proc Natl Acad Sci USA 2003;100:12063–12068.12068 [PubMed: 14519850](b)Edelson B, Best T, Olenyuk B, Nickols N, Doss R, Foister S, Heckel A, Dervan P. Nucleic Acids Res 2004;32:2802. [PubMed: 15155849]
7. (a) Erkkila KE, Odom DT, Barton JK. Chem Rev 1999;99:2777–2795. [PubMed: 11749500] (b) Wang D, Lippard SJ. Nat Rev Drug Discoery 2005;4:307–320. (c) Bruijnincx PCA, Sadler PJ. Curr Opin Chem Biol 2008;12:197–206. [PubMed: 18155674] (d) Metzler-Nolte N. Chimia 2007;61:736–741.
8. (a) Hart JR, Glebov O, Ernst RJ, Kirsch IL, Barton JK. Proc Natl Acad Sci USA 2006;103:15359–15363. [PubMed: 17030786] (b) Zeglis BM, Pierre VC, Barton JK. Chem Commun 2007:4565–4579. (c) Ernst RJ, Song H, Barton JK. J Am Chem Soc 2009;131:2359–2366. [PubMed: 19175313]
9. Brunner J, Barton JK. Biochemistry 2006;45:12295–12302. [PubMed: 17014082]
10. D-Arginine shows improved biostability over the L-enantiomer.
11. (a) Puckett CA, Barton JK. J Am Chem Soc 2007;129:46–47. [PubMed: 17199281] (b) Puckett CA, Barton JK. Biochemistry 2008;47:11711–11716. [PubMed: 18855428]
12. (a) Friedman AE, Chambron JC, Sauvage JP, Turro NJ, Barton JK. J Am Chem Soc 1990;112:4960–4962. (b) Jenkins Y, Friedman AE, Turro NJ, Barton JK. Biochemistry 1992;31:10809–10816. [PubMed: 1420195] (c) Olson EJC, Hu D, Hormann A, Jonkman AM, Arkin MR, Stemp EDA, Barton JK, Barbara PF. J Am Chem Soc 1997;119:11458–11467.
13. (a) Chambron JC, Sauvage JP. Chem Phys Lett 1991;182:603–607. (b) Guo XQ, Castellano FN, Li L, Lakowicz JR. Biophys Chem 1998;71:51–62. [PubMed: 9591359]
14. Copeland KD, Lueras AMK, Stemp EDA, Barton JK. Biochemistry 2002;41:12785–12797. [PubMed: 12379121]
15. Conjugates were purified by reversed-phase HPLC and characterized by MALDI-TOF mass spectrometry. Concentrations were determined by the absorption of $Ru(phen)(bpy')(dppz)^{2+}$; For Ru-D-R8-fluor and Ru-fluor, 361 nm, which is not obscured by fluorescein, was used.
16. HeLa (ATCC, CCL2) were seeded using 4,000 cells in wells of a glass-bottom 96-well plate (Whatman Inc.) and allowed to attach overnight. Imaging was performed using a 63x/1.4 oil immersion objective on a Zeiss LSM 510 or a Zeiss LSM 5 Exciter inverted microscope. The optical slice was set to 1.1 μm. Ru-D-R8 was excited at 488 nm, with emission observed at 560+ nm. For Ru-D-R8-fluor and Ru-fluor, the emission was collected as the combined emission of Ru and fluorescein (505+ nm), both of which are excited at 488 nm.
17. Nakase I, Takeuchi T, Tanaka G, Futaki S. Adv Drug Delivery Rev 2008;60:598–607.
18. Fretz MM, Penning NA, Al-Taei S, Futaki S, Takeuchi T, Nakase I, Storm G, Jones AT. Biochem J 2007;403:335–342. [PubMed: 17217340]
19. Both the fluorescein and the internal carboxylic acid are likely partially deprotonated.
20. Duchardt F, Fotin-Mleczek M, Schwarz H, Fischer R, Brock R. Traffic 2007;8:848–866. [PubMed: 17587406]
21. Kosuge M, Takeuchi T, Nakase K, Jones AT, Futaki S. Bioconjugate Chem 2008;19:656–664.

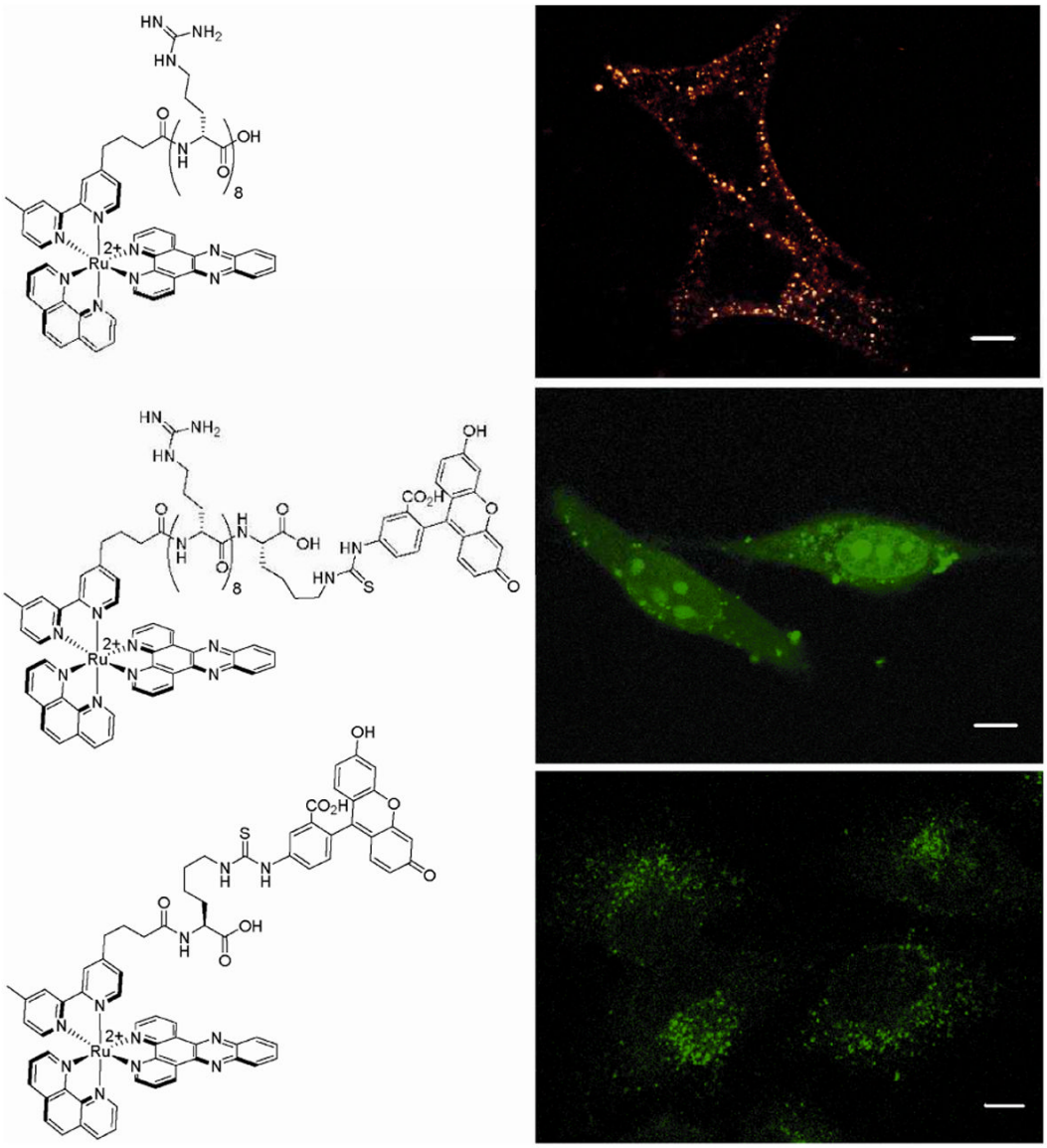


Figure 1.

Typical cellular distribution of Ru conjugates. HeLa cells were incubated with 5 μM Ru-D-R8 for 30 min (top), 5 μM Ru-D-R8-fluor for 30 min (middle) or 20 μM Ru-fluor for 41 h (bottom) at 37 °C in complete medium, then imaged by confocal microscopy. Structures of conjugates are shown at left. Note that Ru-D-R8 is isolated to the cytoplasm while Ru-D-R8-fluor stains the cytosol, nucleus and nucleoli. Ru-fluor shows only weak cytoplasmic staining. Scale bars are 10 μm. See Supplemental for wavelength comparisons.

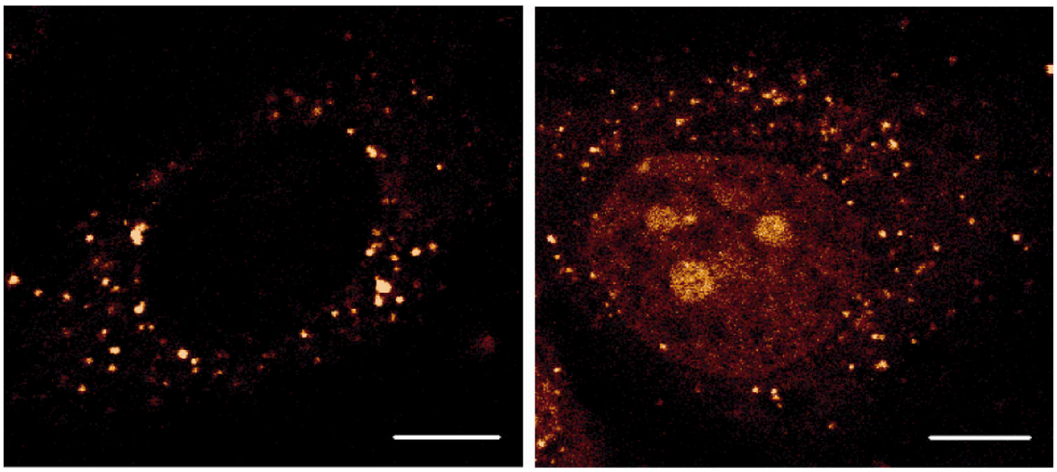


Figure 2.
Cellular distribution of Ru-D-R8 at higher concentration. HeLa cells were incubated with 20 μM Ru-D-R8 for 30 min at 37 °C in complete medium, rinsed with HBSS and imaged by confocal microscopy. The cells shown exclude the membrane-impermeable dead cell dye To-Pro-3. Some cells have only punctate staining of the cytoplasm (left) while others show additional staining of the nucleus and nucleoli as well as diffuse cytoplasmic staining (right). Scale bars are 10 μm.

Table 1

Percentage of HeLa cells with nuclear staining by Ru-octaarginine conjugates[a]

	Ru conjugate	
Conc. (μM)	**Ru-D-R8**	**Ru-D-R8-fluor**
2	0%	0%
5	0%	91%
10	0%	n.d.
15	38%	n.d.
20	60%	n.d.

[a]HeLa cells were incubated with Ru conjugate for 30 min at 37 °C in complete medium, then rinsed with HBSS and analyzed by confocal microscopy. ~50–100 cells were counted for each sample. Dead cells were excluded by their morphology. Data not determined are indicated by n.d.