CHAPTER 3: DIRECTING THE SUBCELLULAR LOCALIZATION OF A RUTHENIUM COMPLEX WITH OCTAARGININE[‡]

3.1: INTRODUCTION

In addition to crossing the cellular membrane, molecular probes and therapeutics must reach their intended location inside the cell. The 5,6-chrysenequinone diimine (chrysi) complexes of rhodium(III) that we are developing as potential chemotherapeutic agents target single base mismatches of DNA.[1–3] Therefore, we are interested in promoting their nuclear accumulation, which should increase their potency and reduce off-target effects.

Confocal microscopy studies on dipyridophenazine (dppz) complexes of Ru(II), luminescent analogues of our rhodium complexes, reveal that they accumulate in the cytoplasm but are predominantly excluded from the nucleus (see Chapter 1).[4] One may surmise, then, that only a fraction of the rhodium(III) chrysi complexes inside the cell are localizing in the nucleus.

A widely used strategy to improve both cellular uptake and nuclear localization is conjugation to a peptide. Cell-penetrating peptides (CPPs), such as the HIV Tat peptide and oligoarginine, facilitate the cellular uptake of many cargos, including peptides, proteins, oligonucleotides, plasmids, and peptide nucleic acids.[5–7] Some CPPs also act as nuclear localization signals (NLSs). Such peptides are rich in positively charged residues such as arginine or lysine and promote active transport through the nuclear pore

[‡] Adapted from Puckett, C. A.; Barton, J. K. Fluorescein redirects a ruthenium-octaarginine conjugate to the nucleus. *J. Am. Chem. Soc.* **2009,** *131,* 8738–8739.

complex.[8] However, the use of peptides is not a fail-proof method for nuclear localization, as entrapment in endosomes can occur, leaving the peptides unable to access the nuclear import machinery.

In earlier work, we 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] As the rhodium complex itself is not fluorescent, fluorescein was attached to monitor the subcellular distribution of this Rh-D-R8 conjugate. However, the potential effects of the fluorescein on the cellular uptake properties cannot be ignored. Some laboratories have varied the fluorescent dye used to assess uptake of a cell-penetrating peptide and found some fluorophore-dependent changes.[10–12] Similarly, the uptake characteristics of pyrrole-imidazole polyamides have been shown to vary with the nature of the appended fluorophore.[13,14]

Luminescent ruthenium(II) polypyridyl complexes allow us to directly observe their subcellular localization, without need of a fluorescent tag. Furthermore, using these complexes, we can isolate the effect of a covalently attached fluorophore on the cellular uptake properties of the metal-peptide conjugate.

3.2: Experimental protocols

3.2.1: Materials and instrumentation

Media, cell culture supplements, Hanks' Balanced Salt Solution, and TO-PRO®-3 iodide were purchased from Invitrogen (Carlsbad, CA).

ESI mass spectrometry was performed at either the Caltech mass spectrometry facility or in the Beckman Institute Protein/Peptide Micro Analytical Laboratory. MALDI measurements were performed on an Applied Biosystems Voyager 6215. Absorption spectra were recorded on a Varian Cary 100 or Beckman DU 7400 spectrophotometer. HPLC was performed on an HP1100 system equipped with a diode array detector using a Vydac C_{18} reversed-phase semipreparative column.

3.2.2: SYNTHESIS OF RU-PEPTIDE CONJUGATES

Peptides, protected and resin-bound, were purchased from Anaspec (Fremont, CA); arginine was protected as its 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) derivative and lysine as its methyltrityl (Mtt) derivative. $Ru(phen)(bpy')(dppz)^{2+}$ was coupled to the peptide in an analogous manner to that previously described (where phen = 1,10-phenanthroline, bpy′ = 4-(3-carboxypropyl)-4′-methyl-2,2′-bipyridine, and dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine).[9,15] Briefly, the acid of the ruthenium complex was coupled to the free N-terminal amine of the peptide by HOBT/HBTU or HATU activated coupling reaction. Fluorescein was added by reaction of fluorescein-5-isothiocyanate (5-FITC) with a lysine residue at the C-terminus. The peptides were cleaved from the resin using 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water for 3 h at ambient temperature and then precipitated by addition of cold diethyl ether. Conjugates were purified by reversed-phase HPLC using a water (0.1% trifluoroacetic acid)/acetonitrile gradient and characterized by MALDI-TOF or ESI mass spectrometry; Ru-octaarginine (Ru-D-R8): 2069.3 *m/z* (M^+) obsd, 2069.4 *m/z* (M^+) calcd,

Ru-octaarginine-fluorescein (Ru-D-R8-fluor): 2585.9 *m/z* (M^+) obsd, 2586.9 *m/z* (M^+) calcd, Ru-fluorescein (Ru-fluor): 668.7 *m/z* (M^{2+}) obsd., 668.7 *m/z* (M^{2+}) calcd. All conjugates employed in this study were used as their trifluoroacetate salts. 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 (ε_{440}= 19,000 M^{-1} cm^{-1}; ε_{361}= 19,469 M^{-1} cm^{-1}).

3.2.3: CELL CULTURE

HeLa cells (ATCC, CCL-2) were maintained in minimal essential medium alpha with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.

3.2.4: CONFOCAL MICROSCOPY

HeLa were seeded using 4000 cells in wells of a glass-bottom 96-well plate (Whatman, Inc.) and allowed to attach overnight. The complexes were incubated with HeLa cells in complete medium (medium with 10% fetal bovine serum) 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. 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. For spectral confocal imaging, HeLa cells were incubated with 10 μM Ru-D-R8-fluor for 60 min at 37 °C, rinsed with HBSS, and analyzed. Emission was collected as a series of bands (10.7 nm width) from 500–720 nm using the multi-channel (META) detector.

3.3: RESULTS AND DISCUSSION

3.3.1: SYNTHESIS OF THE CONJUGATES

Three Ru(II) dipyridophenazine conjugates were synthesized: Ru-octaarginine (Ru-D-R8), Ru-octaarginine-fluorescein (Ru-D-R8-fluor), and Ru-fluorescein (Ru-fluor) (**Figure 3.1**). D-Arginine was chosen for its improved biostability over the L-enantiomer. The conjugates were prepared by solid-phase coupling of $Ru(phen)(bpy')(dppz)^{2+}$ to the N-terminal amine of the peptide. Addition of fluorescein to the C-terminus of the peptide was accomplished via a Mtt-protected lysine, which was selectively deprotected to yield the free ε-amine and reacted with fluorescein-5-isothiocyanate (**Figure 3.2**). For Ru-fluor, a single lysine residue, on solid support, was coupled to the ruthenium complex, followed by addition of fluorescein. Cleavage from the resin can be performed using standard Fmoc cleavage protocols, since the ruthenium complexes are found to be stable under these conditions.[15]

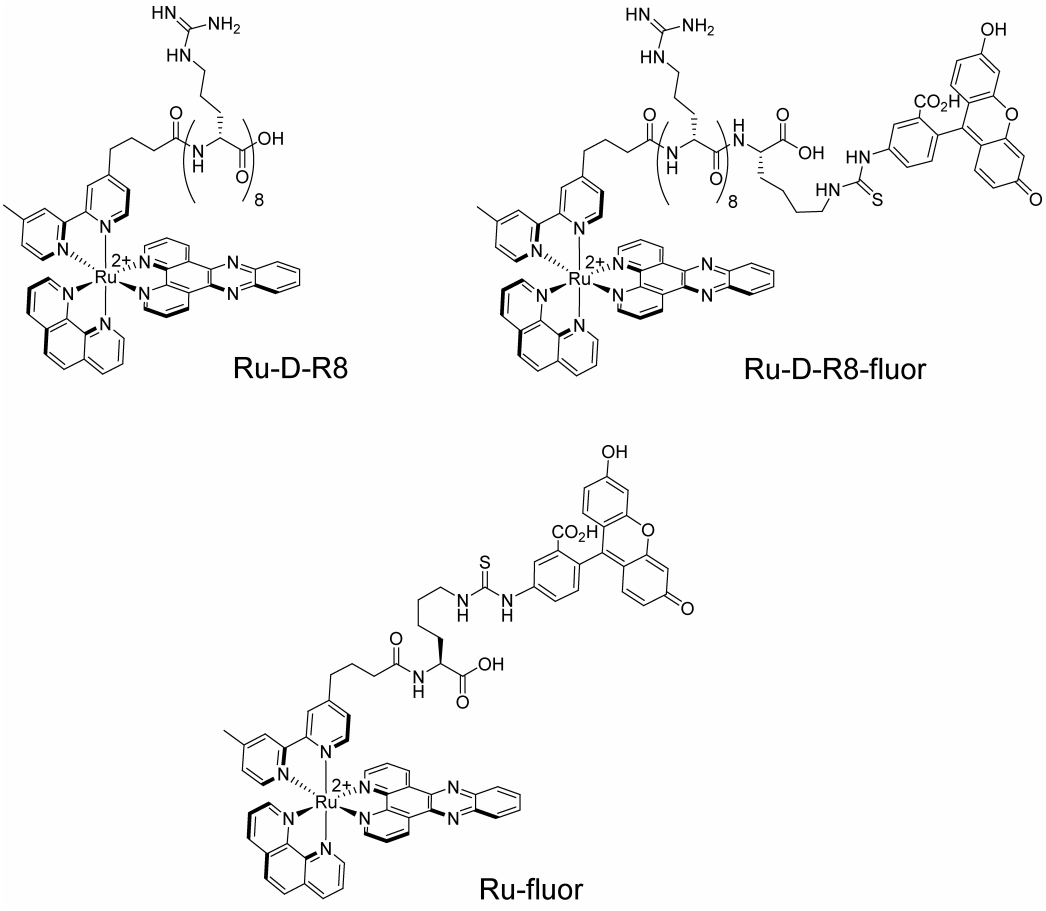

Figure 3.1: Chemical structures of the ruthenium conjugates.

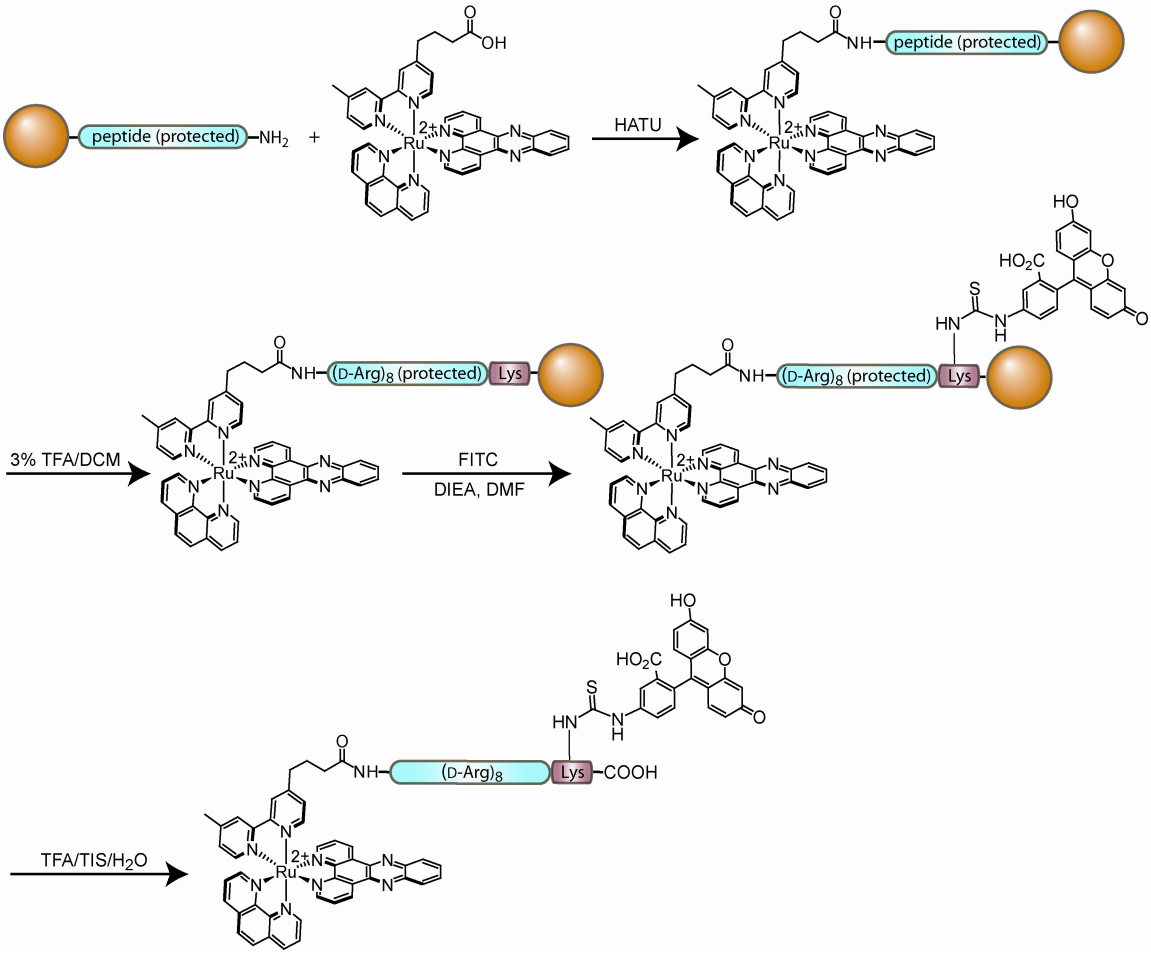

Figure 3.2: Synthesis of Ru-D-R8-fluor. Ru-fluor was synthesized in an analogous procedure using (Fmoc)Lys(Mtt) on the solid support instead of the peptide.

3.3.2: SUBCELLULAR LOCALIZATION OF RU-OCTAARGININE

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 3.3**). The punctate distribution implicates endocytosis, a proposed internalization mechanism for oligoarginine CPPs, as its route into the cell.[16] 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 $Ru(phen)(bpy')(dppz)^{2+}$, $Ru(phen)_2dppz^{2+}$, and $Ru(bpy)_2dppz^{2+}$, which enter by passive diffusion.[17] 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 from 30 min to 2 h or 24 h does not change the subcellular localization of 5 μM Ru-D-R8 (**Figure 3.3**).

At higher concentrations, the distribution 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 3.4**). Nucleolar labeling is typical of D-octaarginine, as seen here, although not of L-octaarginine.[18] The fraction of cells in the latter population increases with concentration (**Table 3.1**). The nucleolar and punctate staining are of similar intensity, with fainter nuclear and cytoplasmic staining.

Population heterogeneity has been described for nonaarginine-fluorescein (R9-fluor).[19,20] What differentiates cells that have greater uptake and nuclear staining versus

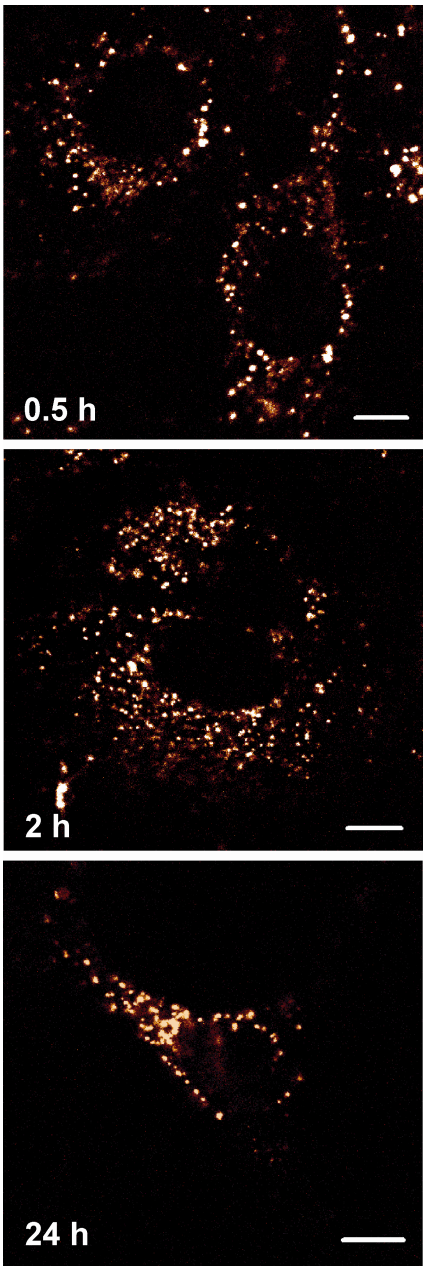

Figure 3.3: Cellular distribution of Ru-D-R8 following different durations of incubation. HeLa cells were incubated with 5 μM Ru-D-R8 in complete medium for 0.5 h, 2 h, or 24 h. The complex is localized in the cytoplasm at all three time points. Scale bars are 10 μm.

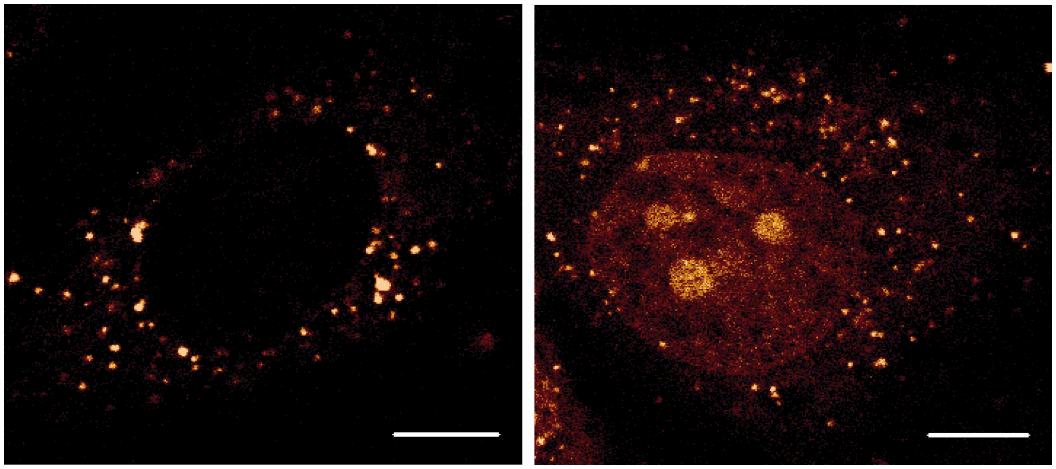

Figure 3.4: Cellular distribution of Ru-D-R8 at higher concentration. HeLa cells were incubated with 20 μM Ru-D-R8 for 30 min at 37 °C in complete medium. The cells shown exclude the membrane-impermeable dead cell dye TO-PRO-3. Some cells have only punctate staining of the cytoplasm (left) while others show additional staining of the nucleus and nucleoli as well as diffuse cytoplasmic staining (right). Scale bars are 10 μm.

Table 3.1: Percentage of HeLa cells with nuclear staining by Ru-octaarginine conjugates[a]

	Ru conjugate	
concn (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.

those that have less is not clear, but these groups of cells do not represent distinct, stable phenotypes. Rothbard and coworkers sorted by flow cytometry the top and bottom 5% of stained cells, re-exposed them to R9-fluor, and re-analyzed them. These cells displayed a similarly broad range of uptake.[19]

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.

3.3.3: Effect of fluorescein on Ru-octaarginine localization

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 3.5**, 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. Notably, at this concentration, D-R8-fluor and the Rh(III) conjugate of D-R8-fluor also localize to the nucleus.[9] The threshold for Ru-D-R8-fluor nuclear entry is between 2 and 5 μM, significantly lower than that for Ru-D-R8 (**Table 3.1**).

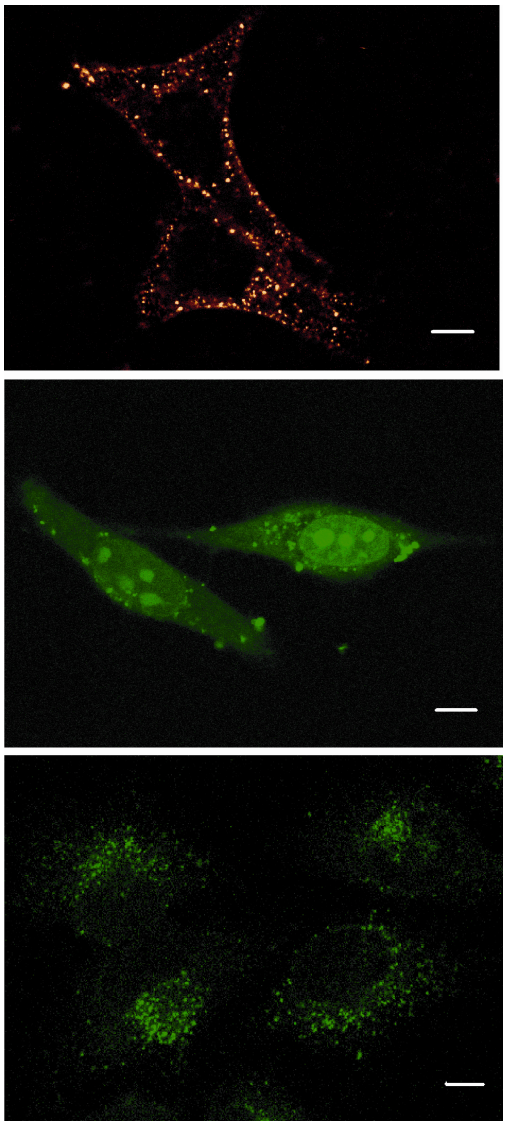

Figure 3.5: Cellular distribution of Ru conjugates. HeLa cells were incubated with 5 μM Ru-D-R8 for 30 min (top), 5 μM Ru-D-R8-fluor for 30 min (center), or 20 μM Ru-fluor for 41 h (bottom) at 37 °C in complete medium. Note that Ru-D-R8 is isolated to the cytoplasm while Ru-D-R8-fluor stains the cytosol, nucleus, and nucleoli. Ru-fluor shows only weak cytoplasmic staining. Scale bars are 10 μm.

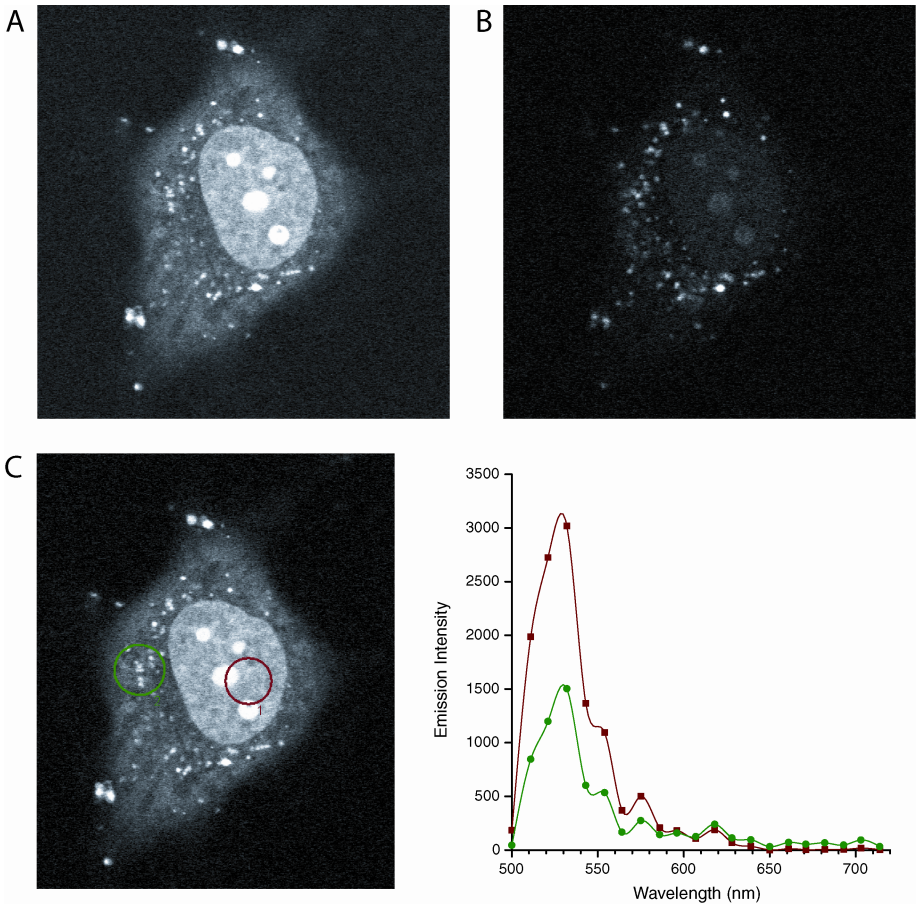

Figure 3.6: Spectral confocal imaging (10.7 nm bandwidth) of HeLa cells incubated with 10 µM Ru-D-R8-fluor for 60 min. (A) Emission at 521 nm. (B) Emission at 618 nm. (C) Emission spectra from nuclear (red) and cytoplasmic (green) regions. Fluorescein (521 nm) and ruthenium (618 nm) emission from the nucleus are apparent.

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 3.5,** bottom). Given its significantly lower positive charge (as both the fluorescein and the internal carboxylic acid are likely partially deprotonated), the complex cannot as effectively use the membrane potential as a driving force for cellular entry.

Spectral confocal imaging of HeLa cells incubated with Ru-D-R8-fluor (10 μM, 60 min) was performed. Emission from both fluorescein (λ_{max} = 521 nm) and Ru (λ_{max} = 618 nm) are observed in the cytoplasm and in the nucleus, which indicates that the conjugate remains intact inside the cell (**Figure 3.6**).

What role is fluorescein playing in the 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.

3.4: CONCLUSIONS

Conjugation of D-octaarginine to $Ru(phen)(bpy')(dppz)^{2+}$ dramatically improves its rate of cellular uptake, reducing the incubation time required to microscopically

observe uptake to less than an hour. At sufficient concentration of conjugate (~ 15 μM), the peptide also increases the nuclear localization; below this threshold concentration only cytoplasmic staining is observed.

This system also allows us to directly observe the effect of a covalently attached fluorescein on cellular uptake properties of the Ru-peptide conjugate. The fluorescein labeled conjugate, Ru-D-R8-fluor, localizes in the nucleus under conditions in which Ru-D-R8 is excluded. Thus, 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.

3.5: REFERENCES

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