Enhanced Cytotoxicity through Conjugation of a "Clickable" Luminescent Re(I) Complex to a Cell-Penetrating Lipopeptide

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Abstract: Re(I) tricarbonyl polypyridine-based complexes are particularly attractive metal complexes in the field of inorganic chemical biology due to their luminescent properties, ease of conjugation to targeting biomolecules, and the possibility to prepare their "hot" (99m)Tc analogues for radioimaging. In this study, we prepared and characterized a novel, "clickable" complex, [Re(2,2'-bipyridine)(3-ethynylpyridine)(CO)3](BF4) ([Re(CO)3(bipy)(py-alkyne)](BF4)), exhibiting the characteristic luminescent properties and moderate cytotoxicity of this general class of compound. Using Cu(I)-catalyzed "click" chemistry, the complex was efficiently attached to a lipidated peptide known to increase cell permeability, namely, the myristoylated HIV-1 Tat peptide (myr-Tat), to give Re-myr-Tat. Fluorescence microscopy localization in human cervical cancer cells (HeLa) confirmed enhanced cellular uptake of Re-myr-Tat compared with [Re(CO)3(bipy)(py-alkyne)](BF4), and cytotoxicity studies showed that this resulted in an increase in potency to a level comparable with cisplatin (13.0 ± 2.0 μM).

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Enhanced Cytotoxicity through conjugation of a “clickable” luminescent Re(I) complex to a cell-penetrating lipopeptide

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Anticancer, Fluorescence Microscopy, Medicinal Organometallic Chemistry, Peptide, Rhenium Complexes

ABSTRACT: Re(I) tricarbonyl polypyridine-based complexes are particularly attractive metal complexes in the field of inorganic chemical biology due to their luminescent properties, ease of conjugation to targeting biomolecules and the possibility to prepare their “hot” 99mTc analogs for radioimaging. In this study, we prepared and characterized a novel, “clickable” complex, \([\text{Re}(2,2’\text{-bipyridine})(3\text{-ethynylpyridine})(\text{CO})_3]\)(BF_4) (\([\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]\)(BF_4)), exhibiting the characteristic luminescent properties and moderate cytotoxicity of this general class of compound. Using Cu(I)-catalyzed “click” chemistry, the complex was efficiently attached to a lipidated peptide known to increase cell permeability, namely the myristoylated HIV-1 Tat peptide (myr-Tat), to give Re-myr-tat. Fluorescence microscopy localization in human cervical cancer cells (HeLa) confirmed enhanced cellular uptake of Re-myr-Tat compared with \([\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]\)(BF_4), and cytotoxicity studies showed that this resulted in an increase in potency to a level comparable with cisplatin (13.0 ± 2.0 μM).

Re(I) tricarbonyl \([2 + 1]\) complexes based on polypyridine-derived ligands, such as \((\text{Re}(\text{bipy})(\text{L})(\text{CO})_3)\) (L = monodentate ligand), have attracted considerable attention in the last decade as catalysts,1 photosensitizers in photocatalytic water reduction2,3 and CO-releasing molecules.4 They remain, however, best known for their outstanding photochemical properties (large Stokes shifts, long emission lifetimes, resistance to photobleaching),5 which make them excellent candidates for cellular imaging and ion sensing applications.6-14 Their use in these areas has been spurred on by their good biocompatibility15 and the possibility to incorporate targeting vectors via the pyridine or bipyridine-based ligands.9, 16, 17 Significantly, the “hot” 99mTc analogs of these Re compounds could also be prepared, making them promising multimodal agents.16,19-20

While Re(I) tricarbonyl polypyridine-based complexes are normally only moderately toxic, or essentially non-toxic, several other Re(I) compounds have been reported to be as active or even more potent than cisplatin.6, 21-26 Given how active many organometallic compounds are known to be against cancer cell lines,20-23 it is surprising that the cytotoxic potential of these Re(I) complexes has not yet been fully explored, especially in light of their aforementioned advantages.

In this work, we aimed to improve the cytotoxicity of a Re(I) compound, namely \([\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]\), whose moderate cytotoxicity was discovered by serendipity in the course of a separate study. To do this, we envisaged enhancing cellular uptake of the complex via conjugation to a myristoylated HIV-1 Tat peptide (Scheme 1). HIV-1 Tat is a membrane translocation sequence from HIV, while myristic acid is a saturated linear fatty acid that naturally occurs as a post-translational protein modification. Both myristic acid and myristoylated Tat peptide were shown to increase the cellular uptake of compounds when conjugated to them.35, 36 In this study, the Re complex was appended to an azide-modified myristoylated Tat peptide via “click” chemistry. Over the past few years, click chemistry has been successfully employed to couple other organometallic compounds to peptides, either by solid-phase or solution-phase methods.37-40

![Scheme 1. Bioconjugation of [Re(CO)₃(bipy)(py-alkyne)]⁺ to a myristoylated Tat peptide.](image-url)
[Re(CO)₃(bipy)(py-alkyne)]⁺(BF₄) was synthesized following an established literature procedure employed for Re(I) fac-tricarbonyl bipyridyl complexes with a substituted pyridine ligand. In brief, the initially formed Re tricarbonyl bipyridyl chloride complex was activated via halide abstraction as the corresponding acetonitrile complex. The acetonitrile ligand was then displaced by 3-ethynylpyridine. The formation and purity of the desired product was confirmed by 'H- and ¹³C-NMR, HR-MS (Figures S1–3) and elemental analysis. Of note, loss of the monodentate pyridine ligand (m/z 427.0 [M-py⁺]) during ionization was observed in the MS spectrum (Figure S3).

Table 1. Emission lifetimes and quantum yields of [Re(CO)₃(bipy)(L)]⁺(BF₄⁻) (25 °C).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Aerated</th>
<th>Degased</th>
<th>Quantum yields (aerated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>117 ± 2 ns</td>
<td>128 ± 3 ns</td>
<td>0.0048 ± 0.0005</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>168 ± 6 ns</td>
<td>329 ± 1 ns</td>
<td>0.011 ± 0.001</td>
</tr>
</tbody>
</table>

Since some studies have previously noted the loss of the monodentate pyridine ligand in solution for this type of complex, the stability of [Re(CO)₃(bipy)(py-alkyne)]⁺(BF₄⁻) in water and human blood plasma was assessed. Following an experimental procedure similar to that previously reported by our group, the complex and diazepam (used as an internal standard due to its known stability in blood plasma and water) were incubated in human plasma or double distilled water for up to 72 h. The aqueous phase was then extracted with dichloromethane and the organic phase analyzed by UPLC-MS. Two peaks corresponding to [Re(CO)₃(bipy)(py-alkyne)]⁺ (1.7 min, m/z 530.1 [M⁺], 427.1 [M-ligand⁺]) and diazepam (2.1 min, m/z 285.2 [M+H⁺]) could be clearly identified (Figure S9–12). The percentage of decomposed [Re(CO)₃(bipy)(py-alkyne)]⁺ was then calculated using diazepam as the internal standard. As shown on Figure 1, decomposition proceeded significantly faster in human blood (half-life of approx. 22 h) than pure water (half-life of approx. 5 days), probably due to the substitution of the pyridine ligand by stoner donor groups present in blood plasma proteins (e.g. histidine or cysteine). These results are consistent with the recent study by the Valliant group, which showed a marked dependence of the pyridine ligand lability on its basicity/leaving group ability. The plasma stability of [Re(CO)₃(bipy)(py-alkyne)]⁺(BF₄⁻) is on par with the most stable compounds reported by Valliant and co-workers.

The azide-modified myristoylated Tat peptide (myr-Tat) was prepared via standard solid-phase peptide synthesis techniques and then purified by preparative HPLC to yield a sticky yellow solid, which was unambiguously characterized by HR-MS and HPLC (Figures S13–14). It was then successfully conjugated to [Re(CO)₃(bipy)(py-alkyne)]⁺(BF₄⁻) via Cu(I)-catalyzed click chemistry using similar experimental conditions to those reported by Fokin and co-workers. The crude compound was purified by preparative HPLC to yield Re-myr-Tat⁺(CO)₃ core in the plasma (water) (double distilled) at 37 °C. The influence of the myr-Tat moiety on the lipophilicity of the resulting bioconjugate Re-myr-Tat⁺ was evaluated by measuring the distribution coefficient between octanol and...
phosphate buffer, pH 7.01 (logD_{7.01}), using a similar procedure to one used by our group for Ru complexes. Interestingly, although \textit{myr-Tat} contains both a long lipophilic fatty acid chain and a highly positively-charged peptide sequence, the net effect is an increase in lipophilicity, namely from a logD_{7.01} of -0.36 ± 0.05 for \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})]^+\) to 0.86 ± 0.15 for \textit{Re-myr-Tat}. This change could potentially improve the cellular uptake of \textit{Re-myr-Tat}, as higher lipophilicity can favor the entry of compounds into cells, and hence enhance the cytotoxicity.

With the desired organometallic complex and bioconjugate in hand, we investigated the intracellular fate of both compounds in human cervical cancer cells (HeLa) by fluorescence microscopy. The cells were first incubated for 2 h with an appropriate concentration of each compound, then fixed with formaldehyde and finally imaged. Figure 2 shows a pronounced difference in emission intensity between the cells treated with \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)^-\) (B) or \textit{Re-myr-Tat} (C). Indeed, while the concentration of \textit{Re-myr-Tat} in the cell medium was five times lower than that of the complex, the cells incubated with \textit{Re-myr-Tat} appeared much brighter, indicating significantly higher uptake (it is important to keep in mind that the intensity of the emission signal of a compound in the cells sometimes fails to correlate with uptake, as luminescence can be quenched in the cellular environment). In terms of subcellular localization, the complex and the bioconjugate were visualized in the cytoplasm (although weak signals were sometimes detected in the nucleoli of cells incubated with the complex) — a localization typically observed for this type of Re complex. Of note, whilst the HIV-1 Tat peptide sequence has been reported to promote nuclear localization, with a myristoylated Tat derivative dual-labeled with Gd-DOTA and fluorescein previously shown to stain both nucleoli and nuclear membranes, this was not the case for \textit{Re-myr-Tat}. Our microscopy images were recorded at a relatively low \textit{Re-myr-Tat} concentration that left most of the cells alive after 2 h. The Gd-DOTA \textit{myr-Tat} derivative, on the other hand, was imaged at a much higher concentration (260 µM), at which about 60% of the cells were already dying. When HeLa cells were incubated with a higher concentration (2.5-fold increase) of \textit{Re-myr-Tat}, localization in nuclear membrane and nucleoli was also observed (Figure S18). However, the cells appeared to be already slightly affected by the conjugate at this concentration, so this change in localization could be due to toxin stress.

Next, the cytotoxicity of \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)^-\) and \textit{Re-myr-Tat} towards HeLa cells was evaluated by incubating the cells with increasing concentrations of the compounds for 48 h and quantifying cell viability using the resazurin assay. The toxicity of our compounds was compared with that of an established metal-containing anti-cancer drug, namely cisplatin. Although several studies report on the cytotoxicity of Re(I) tricarbonyl bipyridine-pyridine complexes, their anti-proliferative effect has generally been found to range from non-existent to moderate; \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)^-\) does not deviate from this trend (Table 2). A series of similar complexes, namely Re(phen)(diaminopy)(CO)$_3$, display IC$_{50}$ values 2–3-fold higher than cisplatin. Significantly, however, the coupling of \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})]^+\) to the myristoylated Tat peptide was found to significantly increase the cytotoxicity of the resulting bioconjugate, bringing it on par with cisplatin.

Table 2. Cytotoxicity data (IC$_{50}$ values) for \([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)^-\), \textit{Re-myr-Tat} and cisplatin towards HeLa cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HeLa IC$_{50}$ (µM)</th>
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<tr>
<td>([\text{Re(CO)}_3(\text{bipy})(\text{py-alkyne})]^+)</td>
<td>29.9 ± 6.1</td>
</tr>
<tr>
<td>\textit{Re-myr-Tat}</td>
<td>13.0 ± 2.0</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>9.1 ± 2.8</td>
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</tbody>
</table>

* Experiments were performed in triplicates.

In conclusion, in this work, we prepared and characterized a new “clickable” Re(I) complex, which could be successfully conjugated to an azide-containing myristoylated Tat peptide to give \textit{Re-myr-Tat}. Cytotoxicity and biological studies with HeLa cells revealed that the anti-proliferative effect of the complex could be enhanced considerably by the addition of a cell uptake-enhancing biomolecule.
ASSOCIATED CONTENT

Supporting Information. Electronic Supplementary Information (ESI) available: characterization of the compounds (NMR, MS, UPLC-MS, UV, emission and IR spectra), emission quantum yields (ESI) available: characterization of the compounds (NMR, MS, UPLC-MS spectra). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

Bipy – bipyridine
DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
ESI – electrospray ionization
HeLa – cervical cancer cell line
HIV – human immunodeficiency virus
HPLC – high-performance liquid chromatography
L – ligand
NMR – nuclear magnetic resonance
HR-MS – high resolution mass spectroscopy
MALDI-TOF – matrix-assisted laser desorption ionization time-of-flight
MLCT – metal-to-ligand charge transfer
Py – pyridine
Tat – “trans-activator of transcription”, here a cell-permeating peptide
UV – ultra-violet-visible light
UPLC – ultra-performance liquid chromatography
UV-vis – ultra-violet-visible light

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peptides with $[\text{Mn(CO)}_3(\text{tpm})]^-$-based CO releasing molecules (tpm = tris(pyrazolyl)methane). *Dalton Trans.* 2009, 4292-4298.


