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Assessing the intracellular fate of rhodium(II) complexes

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Abstract

Rhodium(II)–fluorophore conjugates have strong rhodium-based fluorescence quenching that can be harnessed to report on a conjugate’s cellular uptake and the intracellular decomposition rate.

Information gleaned from this study allowed the design of an improved STAT3 metalloinhibitor.

Graphical Abstract

Transition-metal complexes have a significant history as intracellular medicinal agents, and modern chemical biology increasingly turns to transition-metal complexes as inhibitors,¹⁻⁶ imaging agents,⁷⁻⁹ biological probes,¹⁰ or catalysts with unique properties. When metal complexes must function inside living systems, it is important to understand the cellular uptake and stability of the complex, especially when designing coordination complexes that act on a defined biological target or form coordination bonds with biological targets. Transition-metal complexes can exhibit reactivity, such as ligand exchange and/or redox processes, that are unique from reactions seen with traditional organic molecules. Assessing stability and lifetime of transition-metal complexes inside a living cell is significantly less well understood, despite the importance of this knowledge in designing functional complexes. In this paper, we describe dye-conjugated rhodium(II) complexes that report on the uptake, localization, and stability of rhodium(II) complexes inside living cells, and demonstrate that the information learned can advance the design of STAT3 metalloinhibitors.

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Understanding the stability of metal complexes inside living organisms presents unique challenges. Transition metals can decompose by redox pathways, are strongly sequestered by chaperone and other proteins inside cells, undergo ligand substitution reactions, and are substrates for active transport phenomenon. Methods in common use include relatively crude approaches, such as ICP–MS analysis of homogenized cells,^{11,12} that quantify ion concentration but fail to provide essential information about the coordination environment. Other methods (e.g. MS, HPLC) could potentially measure a specific coordination complex, but sensitivity is often an issue, and many metal complexes of interest do not survive cell homogenation methods.^{11,13} In a few of the most enlightening cases, inherently photoluminescent complexes lose luminescence when degraded, allowing convenient assessment of intracellular stability and localization.^{5,14}

Rhodium(II) complexes have a rich and varied history in biological systems.^{15–17} These complexes interact with DNA, exhibit anti-tumor properties,¹⁸ serve as small-molecule sensors,¹⁷ and have attributes important for photodynamic therapy.^{19–21} Rhodium(II) complexes catalyze reactions of interest for chemical biology applications,^{22,23} and they have been used to inhibit enzyme function.^{24,25} Rhodium(II) complexes have inherently low toxicity,²⁶ though toxicity can be designed with the proper ligand choice.¹⁶ We have recently examined rhodium conjugates with small molecules as inhibitors of protein–protein interactions and other protein functions, where the stability of the metal–ligand linkage is essential. Some rhodium(II) complexes have limited stability under biologically relevant conditions, exhibiting ligand exchange or irreversible reduction.^{27,28} Despite some important successes using fluorescence to track rhodium(II) complexes in living cells,²⁹ better tools to understand the intracellular fate of rhodium(II) complexes, and transition-metal complexes in general, would be broadly useful.

We synthesized fluorophore–rhodium conjugates (e.g. **1b**)—in an attempt to assess cellular uptake—and found that the rhodium conjugates were up to 124 -fold less bright at the wavelength of maximum emission. This behavior is consistent with rhodium-based quenching seen previously for axial coordination to a rhodium center¹⁷ (Fig. 1). Hypochromicity contributes to this reduced fluorescence: the corresponding rhodium complexes have significantly lower molar absorptivity (Figure S2). Conveniently, this observation led to a simple optical probe for the stability and breakdown of the fluorophore–rhodium linkage. Ligand exchange or decomposition pathways, which liberate free fluorophore, result in “turn-on” fluorescence, allowing sensitive measurements of even small amounts of fluorophore release. Validating fluorescence measurements against HPLC analysis verified that “turn-on” fluorescence correlates directly with the release of unbound fluorophore (Figure S4). We probed the stability of these complexes in the presence of various reactive molecules. Reductants, proteins, amino acids, and cellular suspensions were all incubated with **1b** (15 μ M) in 1 $\times$ PBS at 37 °C. As expected, rhodium(II) complex **1b** was reasonably stable under many conditions, but there was a wide range. In the presence of living mammalian cells, complex **1b** was stable almost indefinitely, while high concentrations of TCEP (*tris*(2-carboxyethyl)phosphine) led much shorter half-lives (Fig. 2a). Half-life values were computed by comparison to fluorescence of the parent **1a**, which allowed accounting for photobleaching effects over time.

Although the majority of biomolecules are relatively unreactive toward **1b** (Fig 2a), significant concentrations of reduced thiols resulted in much faster breakdown of the metal complex. Cysteine (200 μ M) itself proved uniquely reactive, producing almost complete breakdown of the rhodium complex in under an hour, faster than did 100-fold higher concentrations of trypsin, which contains several surface-exposed thiols. The process of thiol-mediated decomposition of rhodium(II) centers has been beautifully elucidated previously, involving initial displacement of carboxylate ligands to form a Rh_2S_2 butterfly core with two bridging thiolate ligands.³⁰ These Rh_2S_2 intermediates are highly unstable in an oxygen-containing environment, and readily oxidize to form rhodium(III) products (Fig. 2f).³⁰ A brief screen of redox mediators (Fig. 2b), using complex **3b** as a reporter, identified biological thiols and other reducing agents as likely key mediators of ligand release.

Working with living cells, it was immediately apparent that the rhodium complex **2b** had markedly different penetration than the parent fluorophore **2a**. Live NIH 3T3 cells, dosed with **2a** and **2b**, were rinsed and imaged after 24 h (Fig. 3 a,b). Incorporating a rinse step after the initial incubation ensures that subsequent increases in intracellular fluorescence arise from the breakdown of pre-existing complexes, not from additional complex entering the cells. The fluorescence of **2a**-treated cells is minimal, implying poor cellular entry. In contrast, the rhodium complex **2b** (bottom left) provides bright, high-contrast cell imaging. Because the parent fluorophore (**2a**) does not enter cells efficiently, this result implies that the rhodium complex is cell permeable. Furthermore, the strong image contrast reflects intracellular release of the bright fluorophore upon decomposition of the complex. Similar results are seen with complex **1b**. Cells, treated with **1a** and **1b**, were imaged without rinsing. While **1a** fluoresces indiscriminately (Fig 3c), the cell contrast from **1b** (Fig. 3d) illustrates the visual difference between the metal–dye complex in solution and the released dye inside cells

We were interested in developing rhodium complexes with improved stability, especially in the presence of common intracellular thiols such as glutathione. Terminal carboxamides are, presumably, poorer leaving groups than carboxylates, but, surprisingly, complexes with carboxamide ligands had lifetimes similar to those of simple *n*-alkyl carboxylates. Bulky α,α' -dimethyl substitution at the carboxylate chain to increase steric demand also failed to deliver complexes with increased stability. However, complexation to the sterically demanding carboxylate of fluorescein was significantly more stable (complex **2b**). The increased stability is attributed to the steric screening^(31,32) of an *ortho*-substituted benzoate ligand, as sterically similar 2-phenylbenzoate complexes exhibit similarly increased stability in vitro. In media, the complex **2b** is essentially infinitely stable (Fig. 4a).

The increased stability was reflected in cellular experiments as well. Complexes **1b** and **2b** were incubated with cells for 1 hour, and then rinsed. Cells treated with the simple carboxylate complex **1b** were brightly and maximally fluorescent within 30 minutes, while cells treated with the sterically-demanding fluorescein complex **2b** were mostly dark after 30 minutes (Fig. 4c), but continued to grow in brightness over time. The effect could be quantified by flow cytometry. Mean cellular fluorescence assessed relative to a cell-permeable control dye (**2c**), to account for photobleaching. Cell populations treated with the sterically demanding complex **2b** and rinsed became brighter over time, and the growth in

fluorescence relative to the dye control was a linear function of time over many hours, indicating a long-lived rhodium complex inside the cells (Fig. 4 b).

Intracellular fluorescence turn-on was correlated with intracellular levels of reduced glutathione. The oxidation state of glutathione in living cells, and thus the concentration of reduced thiols, can vary as a consequence of disease state, cell cycle, and in response to external stimuli.³³ In vitro, complex **3b** was incubated with 10-mM glutathione and varying concentrations of *N*-methylethylmaleimide (NEM), a common thiol blocking agent.^{34,35} Increasing NEM leads to a decrease in available free thiol, and this is reflected in lower fluorescence. (Fig. 2c). The effect is also observed in living cells. NIH 3T3 cells were dosed with NEM for 30 min prior to treatment with rhodium complex **1b**. Consistent with hypotheses, the cells without NEM were significantly brighter (Fig. 2 d,e), indicating faster decomposition of the rhodium complex at elevated thiol levels (Fig. 2 d,e).

The speedy breakdown of simple rhodium carboxylates inside living cells has hampered our efforts to design rhodium compounds that inhibit protein-protein interactions like STAT3.^{36–40} The stability concepts discovered here point to a solution to these problems. Naphthalene sulfonamide **4a** binds to the STAT3 protein and inhibits its phosphorylation by upstream kinases in tumor cell lines.⁴¹ We synthesized several rhodium-containing analogues, designed according to principles we developed elsewhere aimed at targeting Lewis-basic side chains.^{42,43} These complexes had generally improved potency in vitro for the disruption of key STAT3 interactions with phosphopeptides, as judged by SPR-based assays. However, this in vitro affinity improvement did not carry over to cellular anti-STAT3 function, presumably due to poor intracellular stability. Simple metalated complexes inhibited STAT3 phosphorylation at levels marginally better than the parent small molecule. Adding a sterically-demanding ancillary ligand to improve intracellular stability did not change in vitro potency (cf. **4b** and **4c**, Fig. 5). However **4c**, demonstrated an improved cellular potency eliciting almost arrest of STAT3 activation (phosphorylation) (Fig. 5).

As a turn-on fluorescent reporter, the method is sensitive to slow initial decomposition rates. The discovery of significantly more stable *ortho*-substituted benzoic acid linkages demonstrates that data from intracellular stability studies can be used to optimize rhodium complex structure. In addition to aiding inorganic biological probe development, evidence indicates that rhodium(II) fluorophores improve the cellular uptake of carboxylate molecules and may have use as reporters of cellular redox state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Rhodium(II) carboxy-fluorophore conjugates. Inset: Emission spectrum of **2a** and **2b** (15 μM in PBS)

Fig. 2.

(a) Half life ($t_{1/2}$) of **1b** in the presence of biologically relevant molecules. The fluorescence data supports that reducing agents, but not oxidants, are the primary mediators of rhodium(II) complex decomposition under biologically relevant conditions. (b) Stability of **3b** toward redox-mediators. Compound **3b** (15 μ M) in PBS was treated with a redox mediator (10 mM) and fluorescence, indicating complex decomposition, was measured after 1h. The red line denotes baseline intensity of the negative control. (c) Addition of *N*-methylmaleimide (NMM) retards decomposition of **3b** and slows the rate of fluorescence

turn-on. **3b** (15 μ M) in 1 $\times$ PBS with GSH (10 mM) and NMM (0–10 mM) (d,e) Cells incubated with **1b** and imaged after 30 min. (f) Proposed formation of an O₂-reactive, bridging thiolate complex upon reaction with glutathione (RSH) (see ref. 29).

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Fig. 3.
Rh-fluorophores are "turn on" imaging agents. Fluorescence of live (a,b) and fixed (c,d) NIH 3T3 cells treated with (a,b) **1a–b** (100 μM) incubated (1 h) and imaged without rinsing. Fluorescent probe (488 nm, green) is shown overlaying cytosol stain (561 nm, red). (c,d) **2a–2b** incubated (100 μM, 30 min) with cells rinsed, fixed, and imaged.

Fig. 4.

(a) $I_{\text{rel}} = I/I_{\text{control}}$ of **1b** and **2b** (15 μM , medium, 37 $^{\circ}\text{C}$) is used to plot $1/[\text{analyte (A)}]$ vs. time, where the control is a combination of **1a** or **2a** + $\text{Rh}_2(\text{OAc})_4$ (b) Fluorescence of **2b** in MOL13-M cells (1×10^5) treated with **2b** (30 μM , 0.5 h) aliquots taken at different times were analyzed by flow cytometry. (c) Fluorescence image of cells (NIH 3T3) 0.5 h after incubation with **1b** or **2b** (100 μM) for 1 h and subsequent rinsing. Complex **2b** showed no signs of cytotoxicity at 30 μM . Fluorescent probe (488 nm, green) is shown overlaying cytosol stain (561 nm, red).

Fig. 5. STAT3 affinity of compounds 4a–c, measured by SPR. Inset: Anti-STAT3 activity of compounds 4a–c (10 μ M) in THP-1 leukemia cells. STAT3 phosphorylation was measured by intracellular flow cytometry 15 minutes after induction of STAT3 phosphorylation with granulocyte-colony stimulating factor (G-CSF, 100 ng/ml). Activity is reported relative to a negative control (“vehicle,” DMSO only).