Cytotoxicity, Cellular Localization and Photophysical Properties of Re(I) Tricarbonyl Complexes Bound to Cysteine and its Derivatives

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Abstract

The potential chemotherapeutic properties coupled to photochemical transitions make the family of $fac-[Re(CO)_3(N,N)X]^{0/+}$ (N,N = a bidentate diimine such as 2,2'-bipyridine (bpy); X = halide, H_2O , pyridine derivatives, PR_3 , etc.) complexes of special interest. We have investigated reactions of the aqua complex $fac-[Re(CO)_3(bpy)(H_2O)](CF_3SO_3)$ (**1**) with potential anticancer activity with the amino acid L -cysteine (H_2Cys), and its derivative N -acetyl- L -cysteine (H_2NAC), as well as the tripeptide glutathione, under physiological conditions (pH 7.4, 37 °C), to model the interaction of **1** with thiol-containing proteins and enzymes, and the impact of such coordination on its photophysical properties and cytotoxicity. We report the syntheses and characterization of $fac-[Re(CO)_3(bpy)(HCys)] \cdot 0.5H_2O$ (**2**), $Na(fac-[Re(CO)_3(bpy)(NAC)])$ (**3**), and $Na(fac-[Re(CO)_3(bpy)(HA)]) \cdot H_2O$ (**4**) using extended X-ray absorption spectroscopy, IR and NMR spectroscopy, electro-spray ionization spectrometry, as well as the crystal structure of $\{fac-[Re(CO)_3(bpy)(HCys)]\}_4 \cdot 9H_2O$ (**2** + 1.75 H_2O). The emission spectrum of **1** displays a variance in Stokes shift upon coordination of L -cysteine and N -acetyl- L -cysteine. Laser excitation at $\lambda = 355$ nm of methanol solutions of **1** – **3** was followed by measuring their ability to produce singlet oxygen (1O_2) using direct detection methods. The cytotoxicity of **1** and its cysteine-bound complex **2** were assessed using the MDA-MB-231 breast cancer cell line, showing that the replacement of the aqua ligand on **1** with L -cysteine significantly reduced the cytotoxicity of the Re(I) tricarbonyl complex. Probing the cellular localization of **1** and **2** using X-ray fluorescence microscopy revealed an accumulation of **1** in the nuclear and/or perinuclear region, whereas the accumulation of **2** was considerably reduced, potentially explaining its reduced cytotoxicity.

1. INTRODUCTION

Despite the great success of platinum-based chemotherapeutics, drugs with a different mode of action other than the platinum antineoplastic family are highly desirable due to the unwanted toxic side effects and prevalence of platinum resistance mechanisms [1]. Treatment of cancer using Photodynamic Therapy (PDT) and Photo-activated Chemotherapy (PACT) has been a rapidly developing area of research due to its multimodal approach [2-4]. In PDT, a nontoxic photosensitizer (PS) is used with light (usually visible/near infrared) and oxygen to generate cytotoxic products inducing localized cell death. PDT initiates apoptosis through different mechanisms which involve the generation of oxidative stress, damaging mitochondria and vascular system, leading to tumour inflammation, and eliciting an immune response against the tumour cells [3, 5, 6].

PACT offers an oxygen-independent alternative to PDT that is particularly useful when considering the characteristic hypoxic environment in cancer cells [7]. Photoactivation can induce ligand substitution reactions in PACT agents, leading to the release of a small cytotoxic molecule such as CO or NO [8-11]. The anti-proliferative effect of CO is thought to be caused by its influence on mitochondria activity in cancer cells, through binding to cytochrome *c* oxidase and inhibiting its activity, generating reactive oxygen species and radicals, and accelerating cellular bioenergetics by high oxygen consumption, thus leading to metabolical exhaustion of the cancer cell [12, 13]. A combined therapy approach can help in circumventing unwanted side effects, such as toxicity and resistance effects associated with some of the currently used chemotherapeutics such as cisplatin [14].

Complexes of transition metals with d^3 and d^6 (low-spin) electronic configurations are great candidates as potential PDT and PACT agents due to their relatively high kinetic inertness and photophysical properties, particularly for d^6 metal complexes that can have a diverse range of possible excited states and light-induced transitions that enhance their photochemical properties [8, 15-18]. For example, Re(I) organometallic compounds and in particular diiminerhenium(I) tricarbonyl complexes $fac-[Re(CO)_3(N,N)X]^{0/+}$ have been studied as potential anticancer therapeutics; for many their cellular distribution could be probed due to their intrinsic luminescence properties, and for some their antiproliferative activity exceeded that of cisplatin [19]. Ford *et al.* demonstrated that irradiation of $fac-[Re(CO)_3(bpy)(P(CH_2OH)_3)](CF_3SO_3)$ (bpy = 2,2'-bipyridine) in the near-UV region ($\lambda = 405$ nm) initiated the photo-induced release of axially coordinated CO, monitoring the intracellular location of the complex using confocal microscopy [20]. Kastl *et al.* introduced a novel class of photosensitizers in the rhenium(I) tricarbonyl complexes that was based on the metallo-pyridocarbazole scaffold, which displayed visible-light-induced ($\lambda \geq 505$ nm) antiproliferative activity [21]. Leonidova *et al.* identified two *N,N*-bis(quinolinoyl) Re(I) tricarbonyl complex derivatives as excellent 1O_2 generators in a lipophilic environment with quantum yields as high as $\sim 75\%$, showing significant enhancement in their cytotoxicity upon UV-A irradiation ($\lambda = 350$ nm) [22].

The rhenium(I) tricarbonyl entity can bind to guanine, forming $fac-[Re(CO)_3(guanine)_2L]^{0/+}$ complexes ($L = H_2O, Br$; guanine = 7-methyl- and 9-methylguanine) with two different conformations: head-to-head (HH) and head-to-tail (HT) around the Re centre that are in equilibrium in solution. This study suggests that similar to cisplatin, $[Re(CO)_3(H_2O)_3]^+$ may target DNA [23]. Cellular targets other than DNA can include metallothioneins (MT), which are cysteine-rich proteins with high affinity for zinc and heavy metals such as Re(I) and Tc(I) [24].

Interaction between $\text{Re}(\text{CO})_3^+$ complexes and MT could be important when treating human breast tumors, as they accumulate unusually high level of zinc that can lead to apoptosis, and over-expression of MT may occur as a method of protection of malignant breast cancer cells from zinc hyper-accumulation [25, 26].

Both *in vivo* and *in vitro* anticancer activities of a series of diimine rhenium(I) tricarbonyl aqua complexes, $\text{fac}[\text{Re}(\text{CO})_3(\text{N,N})(\text{H}_2\text{O})]^+$, have recently been reported [27, 28]. The $\text{fac}[\text{Re}(\text{CO})_3(\text{dmphen})(\text{H}_2\text{O})]^+$ complex (dmphen = 2,9-dimethyl-1,10-phenantroline) showed high affinity for binding to 9-ethylguanine, *N*-acetyl-cysteine and *N*-acetyl-histidine, but not for other amino acids such as methionine, glycine and serine. Cysteine residues of proteins and enzymes could be a potential target and binding site for these complexes. Here, we report the results of our investigations on the reaction products of $\text{fac}[\text{Re}(\text{CO})_3(\text{bpy})(\text{H}_2\text{O})](\text{CF}_3\text{SO}_3)$ (**1**) with *L*-cysteine (H_2Cys), *N*-acetyl-*L*-cysteine (H_2NAC) and glutathione (GSH, denoted H_3A in its triprotonated form), and discuss the impact of such coordination on the cellular localization, cytotoxicity and photophysical properties of the Re(I) complex formed.

2. EXPERIMENTAL SECTION

2.1. Materials

All chemicals and solvents were of reagent grade or higher. Rhenium pentacarbonyl chloride (98%), 2, 2'-bipyridine, tris(2,2'-bipyridine)ruthenium(II) chloride hexahydrate, *L*-cysteine, *N*-acetyl-*L*-cysteine and glutathione were purchased from Sigma Aldrich and used without further purification. Degassed water was prepared by bubbling argon through boiled distilled water until cooled down to room temperature. The oxygen-free aqueous NaOH solution (1.0 M) was prepared by dissolving solid NaOH in degassed water. Dry toluene was prepared by refluxing toluene with

sodium metal and benzophenone under argon [29]. The pH of solutions was measured with a calibrated Thermo Scientific Orion Star pH meter, using standard buffers.

2.2. Cell Culture

MDA-MB-231 breast cancer cells were acquired from the American Type Culture Collection (ATCC) and the passage used within 6 months. Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen/ Gibco) was supplemented with heat-inactivated foetal bovine serum (10% v/v; Sigma Life Sciences), L-glutamine (2 mM, Invitrogen), penicillin (100 units/mL) and streptomycin (100 µg/mL). Cells were incubated at 37 °C in a 5% CO₂-humidified incubator and were sub-cultured every 3–4 days.

2.3. Syntheses

***fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) abbreviated as Re-Aq (1).** The complex was prepared following a previously described method [30], through the synthesis of *fac*-[Re(CO)₃(bpy)Cl]; see *Electronic Supplementary Materials*. A solution mixture of *fac*-[Re(CO)₃(bpy)Cl] (2.69 mmol) and AgCF₃SO₃ (2.69 mmol) in 100 mL acetone was refluxed at 70 °C for 2 h under argon. The white AgCl precipitate was removed by filtering, and the filtrate was concentrated to dryness using a rotary evaporator. The resulting crude yellow solid was recrystallized using an acetone/H₂O (1:3 v/v) mixture and left in a desiccator under vacuum for four days. The yellow crystals formed were filtered and washed with diethyl ether. The unit cell parameters of these crystals were the same as previously reported [31]. Elemental anal. calcd for (*fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) (ReC₁₄H₁₀N₂O₇SF₃): %C 28.33, %H 1.70, %N 4.72. Found: %C 28.43, %H 1.69, %N 4.68; yield 94%. ¹H NMR (600 MHz, D₂O) δ_H 9.14 (d, J = 4.8 Hz, 2H), 8.52 (d, J = 8.2 Hz, 2H), 8.31 (td, J = 7.9, 1.5 Hz, 2H), 7.73 (t, J = 7.0 Hz, 2H). ¹³C {¹H} NMR (600 MHz, D₂O) δ_C 196.9 (CO_{eq}), 192.8

(CO_{ax}), 156.4 (C1, C1'), 154.1 (C3, C3'), 141.4 (C5, C5'), 128.2 (C4, C4'), 124.3 (C6, C6'). IR $\tilde{\nu}_{\text{co}} = 2033, 1914 \text{ cm}^{-1}$.

***fac*-[Re(CO)₃(bpy)(HCys)]·0.5H₂O abbreviated as Re-HCys (2).** Solid *L*-cysteine (2.53 mmol) was added to a completely dissolved, yellow solution of **1** (0.253 mmol) in 20 mL degassed water under argon atmosphere to prevent oxidation of cysteine thiol group. The pH of the solution (4.55) was adjusted to 7.40 by dropwise addition of 1.0 M degassed NaOH, then allowing the orange solution to stir for 48 h at 37 °C. The resulting precipitate was filtered and washed with water. The crude solid, which contained residual sodium triflate by-product was further purified by dissolving it in methanol and filtering the solution. The filtrate was then concentrated to dryness using a rotary evaporator; the orange solid was dried under vacuum at 65 °C with calcium chloride as the drying agent. The electrospray ionization (ESI) mass spectrum of the final product in (-) ion mode did not show a peak at $-m/z = 148.95$ amu for the CF₃SO₃⁻ ion in the final product. Elemental anal. calcd for *fac*-[Re(CO)₃(bpy)(HCys)]·0.5H₂O (ReC₁₆H₁₅N₃O_{5.5}S): %C 34.59, %H 2.72, %N 7.56 (1.62% H₂O). Found: %C 34.25, %H 2.42, %N 7.42 (TG: 1.57% H₂O); yield 58%. Complex **2** is insoluble in water but soluble in methanol; it was initially stable in CD₃OD although some decomposition was observed over a period of seven days. ¹H NMR (600 MHz, CD₃OD) δ_{H} 9.07 (dddd, *J* = 11.2, 5.5, 1.6, 0.8 Hz, 2H), 8.58 (dt, *J* = 8.2, 1.1 Hz, 2H), 8.22 (td, *J* = 7.9, 1.6 Hz, 2H), 7.67 (dddd, *J* = 7.8, 5.5, 2.5, 1.2 Hz, 2H), 3.27 (dd, *J* = 10.7, 3.6 Hz, 1H), 2.99 (dd, *J* = 13.3, 3.7 Hz, 1H), 2.61 (dd, *J* = 13.3, 10.6 Hz, 1H). ¹³C {¹H} NMR (600 MHz, CD₃OD) δ_{C} 198.8 (CO_{eq}), 190.1 (CO_{ax}), 172.3 (C12), 156.0 (C1, C1'), 153.3 (C3, C3'), 139.6 (C5, C5'), 127.6 (C4, C4'), 124.2 (C6, C6'), 59.0 (C11), 31.4 (C10). IR: $\tilde{\nu}_{\text{co}} = 2005, 1907, 1877 \text{ cm}^{-1}$.

Na(*fac*-[Re(CO)₃(bpy)(NAC)]) abbreviated as Re-NAC (3). Following the above procedure, *N*-acetyl-*L*-cysteine (1.68 mmol) was mixed with **1** (0.17 mmol) in 20 mL degassed water under

argon, adjusting the solution pH (2.11) to 7.40. After stirring the mixture for 48 h at 37 °C, the precipitate was removed by filtering, and a small portion of the filtrate was set aside for the ^1H NMR measurement (see below). The crude solid was dissolved in methanol and then filtered; the filtrate was evaporated to dryness. To remove traces of $\text{Na}(\text{CF}_3\text{SO}_3)$, the solid was suspended in water and sonicated for a few minutes. The dark orange solid product (**3**) was filtered and dried under vacuum. It was insoluble in water and displayed poor stability in a range of solvents, undergoing rapid decomposition. Elemental anal. calcd for $\text{Na}(\text{fac}[\text{Re}(\text{CO})_3(\text{bpy})(\text{NAC})])$ ($\text{ReC}_{18}\text{H}_{15}\text{N}_3\text{O}_6\text{SNa}$): %C 35.41, %H 2.48, %N 6.88. Found: %C 35.79, %H 2.54, %N 6.77; yield 54%. IR: $\tilde{\nu}_{\text{co}} = 2003, 1907, 1869\text{cm}^{-1}$. For ^1H NMR measurement, a small portion of the filtrate (when separating the crude solid and solution) was passed through a Sephadex G-15 size exclusion column chromatography to separate unreacted **1** and excess *N*-acetylcysteine. The eluted orange solution was evaporated to dryness and then dissolved in D_2O and further analyzed by ^1H NMR. (400 MHz, D_2O) δ_{H} 9.11 (d, $J = 5.4$ Hz, 2H), 8.52 (d, $J = 8.3$ Hz, 2H), 8.25 (t, $J = 8.1$ Hz, 2H), 7.70 (t, $J = 8.0$ Hz, 2H), 3.98 (dd, $J = 9.3, 3.8$ Hz, 1H), 2.80 (dd, $J = 12.8, 4.0$ Hz, 1H), 2.62 (dd, $J = 13.2, 10.0$ Hz, 1 H), 1.97 (s, 3H).

$\text{Na}(\text{fac}[\text{Re}(\text{CO})_3(\text{bpy})(\text{HA})])\cdot\text{H}_2\text{O}$ abbreviated as Re-GSH (4**)**. Solid glutathione (H_3A ; 1.68 mmol) was added to a completely dissolved solution of **1** (0.17 mmol) in 18 mL degassed water under argon (pH = 2.59). The pH of the solution was raised to 7.40, using 1.0 M degassed NaOH. The orange solution was left stirring for 48 h at 37 °C, and then evaporated to dryness using a rotary evaporator. Methanol was added to the remaining solid, which dissolved the Re-GSH complex but only partially the excess glutathione. The remaining solid glutathione was removed by filtering, followed by evaporation of the filtrate with rotary evaporator. This cycle of adding MeOH, filtering and evaporating was repeated six times. The remaining solid was then dissolved

in 1-2 mL water. Size exclusion column chromatography (Sephadex G-15) was used to further separate excess glutathione from the Re(I)-glutathione product. The orange band on the column was separated, and the resulting solution was again concentrated and passed through the column. This process was repeated three times to ensure separation of unreacted glutathione. The orange solution was rotary evaporated to dryness giving a dark red/orange solid. Despite all the efforts for purifying the sample, the ESI-mass spectrum of the final product still showed a small peak for CF_3SO_3^- ($-m/z = 148.95$ amu; Figure S2), leading to deviations observed in the elemental analysis: calcd for $\text{Na}(\text{fac}-[\text{Re}(\text{CO})_3(\text{bpy})(\text{HA})])\cdot\text{H}_2\text{O}$ ($\text{ReC}_{23}\text{H}_{25}\text{N}_5\text{O}_{10}\text{SNa}$): %C 35.75, %H 3.26, %N 9.06 (2.33% H_2O). Found: %C 32.54, %H 3.60, %N 8.12 (TG: 3.0% H_2O). ^1H NMR (600 MHz, D_2O) δ_{H} 9.07 (dddd, $J = 9.9, 5.5, 1.6, 0.8$ Hz, 2H), 8.48 (dq, $J = 8.5, 1.1$ Hz, 2H), 8.21 (tt, $J = 8.1, 1.3$ Hz, 2H), 7.65 (dddd, $J = 7.7, 5.4, 4.1, 1.2$ Hz, 2H), 4.09 (dd, $J = 8.8, 5.0$ Hz, 1H; C_{11}H), 3.72 – 3.68 (m, 2H; $\text{C}_{13}\text{H}_\text{A}, \text{H}_\text{B}$), 3.63 (d, $J = 17.3$ Hz, 1H; C_{18}H), 2.77 (dd, $J = 13.2, 5.0$ Hz, 1H; $\text{C}_{10}\text{H}_\text{B}$), 2.60 (dd, $J = 13.8, 8.8$ Hz, 1H; $\text{C}_{10}\text{H}_\text{A}$), 2.41 (t, $J = 7.8$ Hz, 2H; $\text{C}_{16}\text{H}_\text{A}, \text{H}_\text{B}$), 2.10 – 2.03 (m, 2H; $\text{C}_{17}\text{H}_\text{A}, \text{H}_\text{B}$). ^{13}C NMR (151 MHz, D_2O) δ_{C} 198.7 (CO_{eq}), 190.5 (CO_{ax}), 176.1 (C_{14}), 174.6 (C_{15}), 173.9 (C_{19}), 172.2 (C_{12}), 155.3 (C_1, C_1'), 153.0 (C_3, C_3'), 139.7 (C_5, C_5'), 127.4 (C_4, C_4'), 123.9 (C_6, C_6'), 57.4 (C_{11}), 54.2 (C_{18}), 43.3 (C_{13}), 31.5 (C_{16}), 30.4 (C_{10}), 26.3 (C_{17}).

2.4. Physical Measurements and Methods

Single crystal X-Ray diffraction. Single crystals of $\{\text{fac}-[\text{Re}(\text{CO})_3(\text{bpy})(\text{HCys})]\}_4\cdot 9\text{H}_2\text{O}$ (**2** + 1.75 H_2O) were grown by using the same reaction conditions as described above. Instead of being continuously stirred at 37 °C, the flask was sealed under Ar and left in the fridge (4° C). After 5 days orange crystals had formed. Single-crystal X-ray diffraction data were collected at 173 K on a Bruker APEX-II CCD diffractometer using graphite-monochromated Cu $\text{K}\alpha$ radiation ($\lambda = 1.5406$ Å). The single crystal was mounted on a nylon Cryo Loops with paratone oil. The data

integration and reduction were processed with the INTEGRATE program of the APEX3 software [32]. Multi-Scan absorption correction was applied to the collected reflections. Using Olex2 [33], the structure was solved with the ShelXT [34] structure solution program using Intrinsic Phasing and refined with the ShelXL [35] refinement package using Least Squares minimization. All non-hydrogen atoms were refined anisotropically. The water hydrogen atoms were located in difference Fourier maps and refined by using the HTAB command. The organic hydrogen atoms were generated geometrically. The refinement parameters are summarized in Table S1.

X-ray absorption spectroscopy (XAS). The Re L₃-edge X-ray absorption spectra were recorded in transmission mode at bending magnetic beamline 9C of the Photon Factory of the High Energy Accelerator Research Organization (Tsukuba, Japan) under dedicated conditions (2.5 GeV with critical energy of 4 keV, 430 - 450 mA). Monochromatization was achieved by means of a Si(111) double crystal monochromator, detuning the beam intensity to 50% at I₀. The first ion chamber (I₀) was filled with nitrogen and the second one (I₁) with (50% N₂+ 50% Ar). The energy scale was calibrated by placing a platinum foil in front of the third ion chamber and assigning its first inflection point to 11564.0 eV. The solid samples **1** – **4** were finely ground and mixed with boron nitride, then mounted in a 1 mm Al frame, using Mylar tape as window material. Three scans were collected for each sample at room temperature.

The extended X-ray absorption fine structure (EXAFS) oscillations were extracted using the WinXAS 3.1 program [36], by subtracting the pre-edge background with a first-order polynomial, followed by edge step normalization. The energy unit was converted to k (Å⁻¹), where $k = [(8\pi^2 m_e / h^2)(E - E_0)]^{1/2}$, using the threshold energy of $E_0 = 10535.7 - 10536.0$ eV for **1** – **4**. A seven-segment cubic spline was then subtracted in the post-edge region to obtain the EXAFS oscillation.

The EXAFS model functions, $\chi(k)$, were constructed with structural information from the crystal structures of $[\text{Re}(\text{CO})_3(\text{bpy})(\text{H}_2\text{O})](\text{CF}_3\text{SO}_3)$ (**1**) and $\{\text{fac-}[\text{Re}(\text{CO})_3(\text{bpy})(\text{HCys})]\}_4 \cdot 9\text{H}_2\text{O}$ (**2** + 1.75 H_2O), using the FEFF 7.0 program to obtain *ab initio* calculated amplitude $f_{\text{eff}}(k)_i$, phase shift $\phi_{ij}(k)$, and mean free path $\lambda(k)$ functions [37, 38].

$$\chi(k) = \sum_i \frac{N_i \cdot S_0^2(k)}{k \cdot R_i^2} \left| f_{\text{eff}}(k) \right|_i \cdot \exp(-2k^2\sigma_i^2) \cdot \exp[-2R_i / \Lambda(k)] \cdot \sin[2kR_i + \phi_{ij}(k)] \quad (\text{eq. 1})$$

Least-squares curve fittings of theoretically simulated EXAFS oscillations $\chi(k)$ to the k^3 -weighted, unfiltered experimental EXAFS spectra were performed over k -range 3.5 – 15.3 $\text{\AA}^{-1}$ (12.8 $\text{\AA}^{-1}$ for **1**), by keeping the coordination number (N_i) constant, and allowing the bond distance (R) and the Debye–Waller parameter (σ^2) to be refined for each backscattering path. The amplitude reduction factor (S_0^2) and ΔE_0 (a common value for all paths) were allowed to float. The accuracy of the bond distances are within ± 0.02 $\text{\AA}$ for Re-S, ± 0.04 $\text{\AA}$ for Re-(C/O/N). Estimated error limits of the Debye-Waller parameters for the Re-C and Re-S paths are ± 0.001 $\text{\AA}^2$; for the Re-(N/O) single scattering and Re-C-O ($n_{\text{leg}} = 3$) multiple scattering paths ± 0.002 $\text{\AA}^2$.

Electronic spectroscopy. UV-vis spectra were recorded using a Cary 50 spectrophotometer. Samples were measured in a quartz cuvette (or in a Schlenk cuvette [39]) with 1 cm path length, using water or methanol as a reference. The molar extinction coefficients (ϵ) of compounds **1** - **3** were evaluated from absorbance data using Beer-Lambert law.

Fluorescence emission spectroscopy. Fluorescence emission spectra were carried out using a Photon Technology International QuantaMaster 400 steady-state spectrofluorimeter (Horiba Scientific). Fluorescence quantum yields of **1** - **3** were determined via a relative method, as described elsewhere [40], using $[\text{Ru}(\text{bpy})_3]\text{Cl}_2$ in air-equilibrated MeOH ($\phi = 0.31$) [41], or quinine sulfate in 0.1 N H_2SO_4 ($\phi = 0.55$) [42] as standards. The absorbance at the excitation

wavelength 290 nm for quinine sulfate and **2**, and at 360 nm for [Ru(bpy)₃]Cl₂, **1** and **3** was kept below 0.05.

¹O₂ Direct Detection. Phosphorescence kinetic traces of ¹O₂ at 1270 nm were acquired with a time-resolved near-infrared (TR-NIR) detection system in air-equilibrated methanol. A diode-pumped Nd:YAG laser (FTSS355-Q3, CryLas) tuned to 355 nm at 1 kHz repetition rate was used as the excitation source for all the complexes. A 1150 nm cut-on long pass filter (FEL1150, ThorLabs) and a 1064 nm notch filter (NF1064-44, ThorLabs) were mounted side by side at the entry port of a monochromator (Digikröm CM110 1/8 m, Spectral Products). A TE-cooled PMT (model H10330A-45, Hamamatsu) working at -908 V was used as the detector at the exit port of the monochromator. The PMT output was amplified to a voltage pulse using a 1.1 GHz preamplifier module (PAM-102-T, PicoQuant GmbH) connected to a multichannel scaler (TimeHarp 260-Nano, PicoQuant). The signal monitored at 1270 nm was collected for 200 s with 256 ns resolution for each time-resolved ¹O₂ emission curves recorded. The time-resolved curves were chi-squared fitted to equation 2 using Prism 7.0 (GraphPad Software Inc.) with the lifetime of ¹O₂ (τ_Δ), the lifetime of the PS (τ_T) and the signal strength (S₀) as free parameters. The goodness of the fittings was assessed by the residual plots. ¹O₂ quantum yields were calculated using tris(2,2-bipyridine)ruthenium(II), [Ru(bpy)₃]²⁺ (φ_Δ = 0.86 in air-equilibrated MeOH) as the reference.[43]

$$S(t) = S_0 \frac{\tau_{\Delta}}{(\tau_{\Delta} - \tau_T)} \left(\exp\left(\frac{-t}{\tau_{\Delta}}\right) - \exp\left(\frac{-t}{\tau_T}\right) \right) \quad (\text{eq. 2})$$

CO Release detection. Possible release of CO gas upon irradiating solutions of **1** - **3** with light (λ = 365 nm) was monitored using UV-vis spectroscopy, and gas chromatography with thermal conductivity detection (GC-TCD) using a solution of *fac*-[Re(CO)₃(bpy)(P(CH₂OH)₃)](CF₃SO₃) in degassed PBS (pH 7.4) as a standard [30]. Solutions of **1**, **2** and **3** (50 μM, 5 mL) in degassed

MeOH were injected in a Schlenk cuvette [39] (internal volume of 23.6 mL) under argon. Each sample was then exposed to $\lambda = 365$ nm light (7.105 mW/cm^2) for two hours, measuring its UV-vis spectrum at 20 minutes intervals. This process was repeated with the GC-TCD method, but after each 20 minutes interval, a gastight syringe was used to remove a 100 μL sample from the headspace of the cell (in Schlenk cuvette) through a special screw-top fitted with a three-layer laminated silicone GC septum. This sample was analyzed by a GC-TCD using an Agilent 6890N gas chromatograph fitted with a Carboxen 1010 PLOT fused silica capillary column ($L \times \text{I.D.} = 30 \text{ m} \times 0.53 \text{ mm}$, average thickness 30 μm). The GC inlet temperature was 230 $^{\circ}\text{C}$. The oven was held at 35 $^{\circ}\text{C}$ for 14 min, then ramped at 20 $^{\circ}\text{C}/\text{min}$ to 245 $^{\circ}\text{C}$, and finally held at 245 $^{\circ}\text{C}$ for 10 min. The retention time for CO is about 11.2 min, and that for CO₂ is 21 min [30]. Oxygen and nitrogen from ambient air have retention times of about 8.2 and 8.4 min, respectively [30]. The ChemStation auto-integration function was used to calculate the peak area for CO.

Cell Viability. The cytotoxicity of complexes **1** and **2**, as well as cisplatin, were measured using the alamarBlue Cell Viability Reagent Assay (Invitrogen). The MDA-MB-231 breast cancer cell line was seeded at 7500 cells per well in black-walled 96-well plates for 48 h at 37 $^{\circ}\text{C}$ in a 5% CO₂-humidified incubator. Fresh stock solutions of **1** and cisplatin were prepared in DMEM whereas for **2**, 1.5% EtOH in DMEM was used as the vehicle. Drug exposure times were 24 and 48 h. Medium was removed using suction and each well was washed with phosphate buffered saline (PBS) before being treated with 10% v/v alamarBlue reagent and left at 37 $^{\circ}\text{C}$ in a 5% CO₂-humidified incubator for 2.5 h. After incubation, fluorescence was measured on a SpectraMax M2e Multi-detection reader using excitation and emission wavelengths of 565-575 nm and 585-595 nm, respectively. Using Prism GraphPad Prism Version 5.03 software, cell viability was determined as a percentage of the fluorescence of cells when treated with **1** or **2** with respect to the control

(DMEM) or vehicle control (1.5% EtOH in DMEM in the case of **2**). By curve fitting-plots of cell viability (%) vs. log of drug concentration, half-maximal inhibitory concentration (IC_{50}) values were calculated. All data were gathered from three independent experiments using 9 biological replicates and their standard deviations were calculated.

X-ray fluorescence microscopy (XFM) - Sample preparation. Stock solutions (800 μ M) of **1** and **2** were prepared in DMEM (solution **2** contained 1.5% EtOH for solubility purposes), and were diluted to 20 μ M with DMEM. As described elsewhere [44], cells were grown on 1.5 mm x 1.5 mm x 500 nm Si_3N_4 windows, by seeding 225,000 cells per well in 6-well plates which were then allowed to attach at 37 °C in a 5% CO_2 -humidified incubator for 24 h. Using 20 μ M solutions of **1** or **2** in DMEM, or only DMEM as control, cells were treated for a period of 6 h. Upon removal of the medium, cells were washed with PBS and then fixed with a 4% paraformaldehyde (prepared fresh in Dulbecco's PBS (D-PBS)) solution for 1 h in an incubator at 37 °C and 5% CO_2 , and then washed with a 100 mM solution of ammonium acetate (in ultrapure water) two times, and air-dried overnight while covered [45, 46].

XFM Data collection and data analysis. Details of data collection and data analysis for XFM have been described elsewhere [44]. XFM images of the cells were obtained at undulator beamline 2-ID-D of the Advanced Photon Source (APS), Illinois, USA, using an incident energy of 12.8 keV using a double crystal monochromator. Using two Fresnel zone plates the beam size was focused to 0.35 μ m; the images were obtained with a spatial resolution of 0.5 x 0.5 μ m in step-scan mode and 1 s dwell time. Using a single element silicon drift energy dispersive detector (Vortex EX, SII Nano-technology, Northridge, CA), X-ray fluorescent spectra were collected from

samples under a He atmosphere set at 75° to the incident beam. Four to five individual cells per sample were selected using an optical microscope.

By fitting the raw fluorescence emission spectra to Gaussians at each spatial point, elemental maps (in units of $\mu\text{g cm}^{-2}$), as well as regions of interest (ROIs), were generated [47, 48]. To obtain Re images, its L_{β} emission line (10010.0 eV) were used for fitting, as its L_{α} line (8586.2 eV) overlap with the Zn K_{α} (8638.9 eV) fluorescence line (Figure S12). Comparison of X-ray fluorescence intensity to those of the thin-film standards, NBS-1832 and NBS-1833, from the National Bureau of Standards (Gaithersburg, MD) allowed for quantification. All data analysis were performed using MAPS software [49, 50].

3. RESULTS AND DISCUSSIONS

3.1. Characterization of Re(I)-thiolate complexes

Using yellow crystals of *fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) (**1**), we successfully prepared three orange/ dark-orange Re(I) tricarbonyl thiolate complexes with bound *L*-cysteinate, *N*-acetyl-*L*-cysteinate or glutathione ligands under physiological conditions (pH = 7.4 and 37 °C): *fac*-[Re(CO)₃(bpy)(HCys)]·0.5H₂O (**2**) and Na(*fac*-[Re(CO)₃(bpy)(NAC)]) (**3**), both of which have poor water solubility, and Na(*fac*-[Re(CO)₃(bpy)(HA)])·H₂O (**4**). These complexes were characterized using ¹H-, ¹³C-NMR (for **2** and **4**), IR and X-ray absorption spectroscopy, as well as electrospray ionization mass spectrometry (ESI-MS). Scheme 1 provides an overview for the syntheses of these Re(I) thiolate compounds.

Single crystals of $\{fac-[Re(CO)_3(bpy)(HCys)]\}_4 \cdot 9H_2O$ (**2** + 1.75 H_2O) were obtained by mixing the reactants in water, adjusting the pH to 7.4 and then leaving the reaction mixture at 4 °C (see the *Experimental Section*). After 5 days small orange crystals had formed which were suitable for single crystal X-ray diffraction (see Figure 1). Crystallographic data, and selected interatomic distances and bond angles are reported in Tables S1 and S2. The protonation state of the carboxylate ($-COO^-$) and the amine (as $-NH_3^+$) groups in the crystal structure of $\{fac-[Re(CO)_3(bpy)(HCys)]\}_4 \cdot 9H_2O$ was determined based on the neutrality of the complex, its elemental analysis, and the fact that there was no counter ion (e.g. Na^+ or $CF_3SO_3^-$) present in the unit cell. Since the complex was crystallized at pH 7.4, formation of $(H_3O)[Re(CO)_3(bpy)(Cys)]$ or $[Re(CO)_3(bpy)(H_2Cys)](OH)$ is not feasible.

Scheme 1. Synthesis of $fac-[Re(CO)_3(bpy)(H_2O)](CF_3SO_3)$ (**1**) and its subsequent reaction products (**2** – **4**) with thiol containing biomolecules *L*-cysteine, *N*-acetyl-*L*-cysteine, and glutathione. The numbers in red denote the carbon atoms associated with the ^{13}C NMR signals described in the *Experimental Section*.

Figure 1. Crystal Structure of $\{fac-[Re(CO)_3(bpy)(HCys)]\}_4 \cdot 9H_2O$ (**2** + 1.75 H_2O). Water molecules have been omitted for clarity.

A survey of the Cambridge Structural Database (CSD) [51] shows that the Re-S bond distances of 2.503(6) and 2.496 (10) Å in the two *fac*-[Re(CO)₃(bpy)(HCys)] entities are similar to the average Re-S distance in Re(CO)₃(*N,N*)⁺ complexes with S-donor ligands: 2.509 ± 0.023 Å (Table S3). However, the mean Re-C_(axial) bond length (1.925 ± 0.005 Å; Table S3) in these complexes is slightly longer than the corresponding distances (1.879(24) Å and 1.909 (20) Å) in the Re(I) cysteinate complex **2**, and also longer than the reported Re-C_(axial) distance in the Re-Aq complex (**1**) 1.882(10) Å [31].

The k^3 -weighted Re L₃-edge X-ray absorption fine structure (EXAFS) spectra for complexes **1** – **4**, and their corresponding Fourier transforms (FTs) are shown in Figure 2. The FTs of the Re(I)- thiolate complexes (**2** – **4**) display a small peak at ~ 2.2 Å (not corrected for phase shift) associated with the Re-S scattering that is absent in the FT of the Re-aqua complex **1**, *fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃). This difference shows that contradictory to an earlier report [52], Re L₃-edge EXAFS spectroscopy would be sensitive to the nature of the axial ligand X in *fac*-[Re(CO)₃(*N,N*)X]^{0/+} complexes, if high quality data ($k > 11$ Å⁻¹) are obtained.

Comparing the average bond distances in the first coordination sphere of Re(I) in **2** obtained from EXAFS data analysis (Re-C 1.93 Å, Re-N 2.18 Å, Re-S 2.48 Å; Table 1), with those from its crystal structure (1.90 Å, 2.14 Å, 2.50 Å, respectively) shows that the accuracy of these distances is within ± 0.02 Å for Re-S and ± 0.04 Å for Re-(C/N) bonds.

The Debye-Waller parameter (σ^2) values for the Re-C and Re-(N/O) scattering paths in the EXAFS fitting model of **1** seem reasonable (0.0021 ± 0.001 Å² and 0.0055 ± 0.002 Å², respectively; Table 1), while that of the longer Re-C-O ($n_{\text{leg}} = 3$) path is relatively small (0.0022 ± 0.002 Å²). In the EXAFS model fitting of Re(CO)₃ bound to a TiO₂ surface, a similar σ^2 value was reported for the corresponding multiple scattering path [53]. By replacing H₂O with a cysteinate ligand in **2**, and introducing a Re-S path in its EXAFS model fitting, an unreasonably small σ^2 value was obtained for the Re-N path (Table 1), which is probably due to the interference between their EXAFS oscillations (see Figure S1).

Figure 2. k^3 -weighted model fitted Re L₃-edge EXAFS spectra of Re(I) complexes **1** – **4**, and their corresponding Fourier transforms (see Table 1).

Table 1. Least Squares Curve-fitting Results for Re L₃-edge EXAFS Spectra of Solids **1** – **4** (see Figure 2) ^a

	Re-C			Re-(N/O)			Re-C-O (n _{leg} =3)			Re-S			S ₀ ²	ΔE ₀	$\mathcal{R}$
	<i>N</i>	<i>R</i>	σ ²	<i>N</i>	<i>R</i>	σ ²	<i>N</i>	<i>R</i>	σ ²	<i>N</i>	<i>R</i>	σ ²			
1	3 <i>f</i>	1.88	0.0021	3 <i>f</i>	2.12	0.0055	6 <i>f</i>	3.05	0.0022				1.08	1.7	32.9
2 ^b	3 <i>f</i>	1.93	0.0037	2 <i>f</i>	2.18	0.0005	6 <i>f</i>	3.08	0.0023	1 <i>f</i>	2.48	0.0029	0.94	8.6	21.8
3	3 <i>f</i>	1.91	0.0029	2 <i>f</i>	2.16	0.0027	6 <i>f</i>	3.08	0.0025	1 <i>f</i>	2.47	0.0078	0.95	7.1	28.7
4	3 <i>f</i>	1.92	0.0027	2 <i>f</i>	2.17	0.0010	6 <i>f</i>	3.08	0.0023	1 <i>f</i>	2.48	0.0037	0.95	7.4	24.7

^a *f* = fixed, $\mathcal{R}$ = fitting residual; the accuracy of the average distances is within ± 0.02 Å for Re-S and ± 0.04 Å for Re-(C/N/O). ^b See the contribution of each scattering path in the model fit in Figure S1.

The Re-GSH complex (**4**) shows a very similar first coordination sphere as the Re-HCys complex (**2**) with comparable average bond distances obtained from the EXAFS data analysis; see Table 1. The ESI-mass spectra of this complex, in both (-) and (+) ion modes, displayed intense peaks at $-m/z = 732.08$ and $+m/z = 756.07$ amu $\{[\text{Re}(\text{CO})_3(\text{bpy})(\text{HA})]^- + \text{Na}^+ + \text{H}^+\}^+$, indicating that $[\text{Re}(\text{CO})_3(\text{bpy})(\text{HA})]^-$ is the dominating species in this sample (see Figure S2 and Table S4).

The ¹³C NMR spectra of both Re-HCys (**2**) and Re-GSH (**4**) complexes displayed a signal for the Cys C_β atom (C₁₀) at δ_c = 31.4 (in CD₃OD) and 30.4 ppm (in D₂O), respectively, being somewhat deshielded relative to those of the pure ligands in D₂O (pD = 7.8 [54], reading pH = 7.4): δ_c = 26.6 and 26.0, respectively (see Figures S3 (a) and S4 (a)). This downfield shift of Δ(δ_c) ~ 4 – 5 ppm is associated with the coordination of their cysteine thiolate groups to the Re(I) ions. Similar shifts have been previously observed for Zn(II), Hg(II), Pb(II), Cd(II) ions bound to cysteine and glutathione [55]. The ¹H NMR signals, however, of the two hydrogen atoms (H_A, H_B) on the Cys C_β atoms (C₁₀) in pure cysteine and glutathione (δ_H = 2.9 – 3.0 ppm; pD = 7.8 in D₂O)

showed an upfield shift of $\Delta(\delta_H) \sim 0.3 - 0.4$ ppm when bound to Re(I) ions in complexes **2** and **4** (see Figures S3 (a) and S4 (a)). This shift could be explained by the shielding effect imposed by the bipyridine ring on the C₁₀ hydrogen atoms (H_A and H_B). As shown in the crystal structure of **2** (Figure 1), the side chain of the cysteinate ligands adopt a “folded” conformation, where these C₁₀ hydrogen atoms (H_A and H_B) get close to, or are stacked above the bipyridine aromatic rings. Similar intramolecular interaction and folding of the axial ligand’s extended chain over the bipyridine ring has previously been observed for the $[\text{Re}(\text{CO})_3(\text{bpy})\text{NC}(\text{CH}_2)_n\text{CH}_3]^+$ complex ions [56].

The characteristic C≡O vibrational stretching bands for the Re-HCys complex (**2**) appeared at lower frequencies in the FT-IR spectra relative to those of the Re-Aq complex (**1**). For example, the totally symmetric in-phase $\nu(\text{CO})$ vibrational resonance, denoted as A'(1), appeared at 2033 and 2005 cm⁻¹ for **1** and **2**, respectively. Similar shifts to lower wavenumbers were observed in the asymmetric vibrational frequency of the equatorial CO ligands (A'' mode, 1907 cm⁻¹) and the totally symmetric out-of-phase $\nu(\text{CO})$ vibration (A'(2) mode, 1877 cm⁻¹); see Figure S5 [57]. The shift of the C≡O stretching vibrations to lower frequencies for **2** could be attributed to the increased electron density on Re(I) ion from the coordinated thiolate group, which is then back-donated to the π^* orbital of carbonyl ligands, making the C≡O bond weaker [57].

As for the Re-NAC complex (**3**), small ESI-mass peaks could be observed at $-m/z = 588.02$ amu and $+m/z = 590.04$ amu, which were attributed to $[\text{Re}(\text{CO})_3(\text{bpy})(\text{NAC})]^-$ and its protonated form $[\text{Re}(\text{CO})_3(\text{bpy})(\text{NAC}) + 2\text{H}^+]^+$, respectively (see Figure S2 and Table S4). In the FT-IR spectrum of **3**, the carbonyl stretching bands $\nu(\text{CO})$ appeared at similar frequencies as those of the Re-HCys complex (**2**): 2003 cm⁻¹ (A'(1)), 1907 (A''), and 1869 cm⁻¹ ((A'(2))) (see Figure S5). EXAFS data analysis of this complex, however, showed that the mean Re-S distance has relatively

high variation ($\sigma^2 = 0.0078 \pm 0.0010 \text{ \AA}^2$; Table 1) compared with complexes **2** and **4** ($\sigma^2 = 0.003 - 0.004 \text{ \AA}^2$). A larger σ^2 value for the Re-S bond in **3** reflects a larger structural disorder, or vibrational disorder along the Re-S bond, relative to **2** and **4**, even though all three compounds have similar mean Re-S distances ($2.47 - 2.48 \text{ \AA}$; Table 1). For similar type of bonds (e.g. Re-S), there is a correlation between the bond length and vibrational frequency, force constant and therefore bond strength: The longer the bond, the lower the vibrational frequency; the force constant would be smaller and the bond would be weaker. Thus, Re-S bonds with a higher vibrational/ structural disorder may indicate a weaker Re-S bond in the Re-NAC complex (**3**) than in **2** and **4**. It would also explain the rapid decomposition (i.e. ligand (NAC^{2-}) – solvent exchange) of **3** in CD_3OD (Figure S6), and other organic solvents (CD_3CN , CDCl_3 , CD_3COCD_3), while the Re-HCys complex (**2**) was stable in d^4 -methanol. Therefore, the small structural difference between cysteine and *N*-acetylcysteine appears to reflect a significant effect on the overall stability of the complex. It is noteworthy that based on previous NMR data of **1** in CD_3OD , the *fac*- $[\text{Re}(\text{CO})_3(\text{bpy})(\text{H}_2\text{O})]^+$ complex is able to establish an equilibrium in the ligand exchange between H_2O and CD_3OD , establishing an equilibrium, as evidenced by observing two sets of bipyridine peaks in the aromatic region. The addition of D_2O shifted the equilibrium to the intended product of **1**, observed through coalescence of the signals [27].

3.2. Photophysical Properties

The three pure Re(I) complexes $[\text{Re}(\text{CO})_3(\text{bpy})\text{X}]^{+/0/-}$ ($\text{X} = \text{H}_2\text{O}$ (**1**), HCys^- (**2**), NAC^{2-} (**3**)) were further characterized by recording their UV-vis absorption, excitation and emission spectra, and evaluating their fluorescence quantum yield (Φ_F) and $^1\text{O}_2$ quantum yield (Φ_Δ) in air-equilibrated MeOH at 298 K (Table 2). Absorbance data of **1** was previously reported in DMF showing the absorption and emission maxima at $\lambda_{\text{max}} = 350 \text{ nm}$ and $\lambda_{\text{em}} = 540 \text{ nm}$, respectively [58].

Table 2. Photophysical Properties of Complexes **1 - 3**

Complex	$\lambda_{\text{max}} (\epsilon) / \text{nm} (10^4 \text{ M}^{-1} \text{ cm}^{-1})^a$	$\lambda_{\text{em}}^b / \text{nm}$	Φ_{F}^b	$\Phi_{\Delta} (\%)^c$
1	243 (1.8), 297 (1.32), 303 (1.3), 317 (0.935), 356 (0.325)	587	0.037 ± 0.004	15.0 ± 1.8
2	243 (1.8), 291 (1.25), 370 (0.226), 475 (0.05)	458	0.0010 ± 0.0002	1.2 ± 0.1
3	244 (2.0), 291 (1.41), 360 (0.33), 484 (0.055)	608	0.007 ± 0.001	8.5 ± 0.9

^a Electronic absorption spectra were measured in MeOH at room temperature; λ_{max} = maximum absorption; molar absorption coefficient (ϵ) values were determined by measuring the spectra of each complex at different concentrations (see Figure S7). ^b In air-equilibrated MeOH at 298 K; λ_{em} = maximum emission; Φ_{F} = fluorescence quantum yield. ^c Φ_{Δ} = $^1\text{O}_2$ quantum yield in air-equilibrated MeOH at 298 K.

The electronic absorption spectra of complexes **1**, **2** and **3** display an intense band at 243 nm (Figure 3), which is independent of the type of axial ligand X. It has been attributed to the bipyridine localized intra-ligand (^1IL) $\pi \rightarrow \pi^*$ transition [59, 60]. Another intense absorption band was observed in the 290-300 nm region, similar to that of *fac*-[Re(CO)₃(bpy)(pyridine 4-thiolate)] in CH₂Cl₂ at 295 nm, which was assigned by the authors to $^1\text{IL } n \rightarrow \pi^*$ transition in the aromatic ligands [61]. However, time-dependent density functional theory (TD-DFT) calculations of singlet electronic transitions for *fac*-[Re(CO)₃(bpy)Cl] showed that its high-energy, strong absorption bands < 350 nm mainly originate from $^1\text{IL } \pi \rightarrow \pi^*$ [62, 63]. Stonayov et al. assigned its peak at 292 nm (solvent = 4:1 v/v EtOH: MeOH) to ligand-centered $\pi \rightarrow \pi^*$, [62] while El-Nahas et al. attributed its absorption peaks at 293 and 317 nm (solvent = DMF) to Re(CO)₃Cl $\rightarrow$ bpy transitions [63], originating from HOMO-1 and HOMO-2 with mixed Re ($d\pi$), CO (π^*) and Cl ($p\pi$)

contributions, and LUMOs mainly having bpy π^* character [57, 62]. In the present study, the change in the axial ligand X has caused a blue shift in the peak position from 297 nm for **1** (X = H₂O) to 291 nm for **2** and **3** (X = HCys⁻, NAC²⁻), where there is higher electron density on the Re(I) ion from the electron-donating thiolate (⁻SR) ligands. As such, Re(CO)₃(SR) → bpy transitions may contribute in this band.

Figure 3. UV-vis absorption spectra of the Re(I) complexes **1** – **3** in MeOH ($C_{\text{Re}} = 5.0 \times 10^{-5}$ M); inset presents details in the 325 – 600 nm region.

There are also less intense, low-energy absorption bands in the 325 – 550 nm region, originating from several ¹CT (singlet charge-transfer) transitions. Complex **1** shows a broad absorption feature with $\lambda_{\text{max}} = 356$ nm (tailing to ~ 480 nm). A similar absorption was observed for *fac*-[Re(CO)₃(bpy)Cl] at $\lambda_{\text{max}} = 370$ nm (tailing to ~ 430 nm), which based on TD-DFT calculations was assigned to Re(CO)₃Cl → bpy ¹CT transition (HOMO-1 → LUMO (bpy π^*)) [63,

64], also described as metal-ligand to ligand charge transfer (¹MLLCT).[62] The stabilizing effect of the coordinated water molecule to Re(I) ion on the HOMO energy can explain the hypsochromic shift of the absorption maximum observed for complex **1** compared to *fac*-[Re(CO)₃(bpy)Cl].

Substitution of H₂O with cysteinate ligand leads to significant changes in the 325 – 550 nm region. Instead of a clear band, complex **2** displays a shoulder at ~ 370 nm, which is followed by two broad weak absorptions in the visible region at ~ 475 and ~ 520 nm. These changes are reminiscent of the ones observed for rhenium(I) tricarbonyl complexes with arenethiolate axial ligands [65]. As reported earlier, these low-energy bands are responsible for the orange-red color of the Re(I) thiolate complex compared to the yellow complex **1**, and have been assigned to another charge transfer state [65]. Interestingly, Figure 3 shows that the Re(I)-NAC complex (**3**) has absorption features ($\lambda_{\text{max}} = 360, 484 \text{ nm}$) between those of **1** and **2**, indicating that solvent molecules can replace the NAC²⁻ ligands, forming *fac*-[Re(CO)₃(bpy)(CH₃OH)]⁺ (eq. 3):

This is another indication that complex **3** is unstable in MeOH, which is consistent with our earlier observation from its ¹H NMR spectrum in CD₃OD (Figure S6).

Emission and excitation spectra of the Re(I) complexes **1**, **2** and **3** were also measured in air-equilibrated MeOH at 298 K (Figure 4). The emission spectrum of the *fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) complex (**1**) displayed an intense, structureless broad peak ($\lambda_{\text{em}} = 587 \text{ nm}$) that is consistent with the typical emission of diimine Re(I) tricarbonyl complexes and has been ascribed to originate from a triplet metal-to-ligand charge-transfer (³MLCT) states.[27] Upon substitution of H₂O with the cysteinate ligand, the maximum emission for the Re-HCys complex (**2**) appears at $\lambda_{\text{em}} = 458 \text{ nm}$. This complex is almost non-emissive as indicated by the

extremely low fluorescence quantum yield (Table 2), which is associated to a very short fluorescence lifetime (Table S5). While surprising, this result is not unprecedented as a similar effect has previously been observed for the emission properties of an arenethiolate complex, *fac*-[Re(CO)₃(Pyta)(SPhOCH₃)], where Pyta = 4-(2'-pyridyl)-1,2,3-triazole, and SPhOCH₃ = 4-methoxybenzenethiolate ($\lambda_{\text{em}} = 450 \text{ nm}$).^[65] The emission bands for similar arenethiolate complexes appeared at lower energies, implying that they were highly dependent on the nature of the thiolate and diimine (*N,N*) ligands. Based on DFT calculations, He et al. suggested that these emissions may have a mixed ³MLCT/ ³LLCT (ligand-to-ligand charge transfer) character, originating from a π (S/benzenethiolate)/ Re ($d\pi$) $\rightarrow \pi^*(N,N)$ transition.^[65] Similarly, Fernández-Moreira et al. proposed that the low intensity emission of *fac*-[Re(CO)₃(bpy)(pyridine 4-thiolate)] ($\lambda_{\text{em}} = 572 \text{ nm}$) could be assigned to the ³MLCT transition, involving a possible non-radiative secondary process (LLCT).^[61]

Our data suggest that the weak emission of **2** mainly originates from the MLCT as indicated by the excitation spectra which are centered around the same wavelengths for all the complexes (Figure 4). It has been shown for a series of [Re(CO)₃(*N,N*)(L)]⁺⁰ complexes that the higher the electron density on the Re(I) ion, the lower the energy of the ³MLCT emission band.^[66, 67] Since the CO stretching vibrations of **2** appeared at lower frequencies than those of **1** (Figure S5), the electron density on the Re(I) ion is higher in **2** (from the π -donor *S*-cysteinate ligand). Therefore the ³MLCT transitions for **2** are expected to occur at lower energies/ higher wavelengths than those of **1** ($\lambda_{\text{em}} = 587 \text{ nm}$). However, the experimental emission spectrum of **2** shows the opposite ($\lambda_{\text{em}} = 458 \text{ nm}$), having a very short fluorescence lifetime (Table S5). Taken together, these data suggest that the emission of Re-HCys complex **2** could potentially originate from the ¹MLCT, rather than

the typical $^3\text{MLCT}$. The exact assignment of the emission band for **2** requires further theoretical calculations that is beyond the scope of this study.

The excitation spectrum of **2** exhibits vibronic bands that are associated with the bipyridine ligand. Indeed, the difference in wavenumbers of the peak maxima are in the range of $1077 - 1246\text{ cm}^{-1}$, which corresponds to in-plane ring stretching and C-H in-plane bending of the bipyridine [68].

Figure 4. Excitation (solid line) and emission (dashed line) spectra of **1 - 3** in air-equilibrated MeOH at 298 K . (Excitation wavelength: $\lambda_{\text{ex}} = 360\text{ nm}$ for **1** and **3**, 290 nm for **2**)

Interestingly, the emission spectrum of the Re-NAC complex (**3**) displayed two luminescence bands (Figure 4): a low intensity broad peak centered at 442 nm , which is close to

the maximum fluorescence observed for the Re-cysteinate complex (**2**), and an intense broad peak at 608 nm that is red-shifted relative to the $^3\text{MLCT}$ phosphorescence of the Re-Aq complex (**1**), and can be assigned to the *fac*- $[\text{Re}(\text{CO})_3(\text{bpy})(\text{CH}_3\text{OH})]^+$ species. Observing these two emission bands for **3** is consistent with its electronic absorption features being in-between those of **1** and **2** (Figure 3), and the complexity of its ^1H NMR spectrum in CD_3OD due to ligand (NAC^{2-}) - solvent exchange (eq. 3; Figure S6). The red shift of the $^3\text{MLCT}$ band to 608 nm could be explained by the higher electron density on Re(I) ion in **3** [66], since CH_3OH is a slightly better σ -donor than H_2O due to the inductive effect of the methyl group. Furthermore, the slight increase in the fluorescence quantum yield of **3** ($\Phi_{\text{F}} = 0.007 \pm 0.001$) could be related to generation of *fac*- $[\text{Re}(\text{CO})_3(\text{bpy})(\text{CH}_3\text{OH})]^+$ in MeOH solution, as discussed earlier (eq. 3).

Singlet Oxygen Quantum Yield. We further examined the ability of the complexes **1**, **2** and **3** to produce $^1\text{O}_2$ using direct detection method, i.e. by exciting the samples with a 355 nm laser source and measuring the near-IR luminescence of $^1\text{O}_2$ at 1270 nm (see Figure S8). Singlet oxygen quantum yields for **1** – **3** could only be obtained in air-equilibrated MeOH (Table 2). No signal could be detected for **1** – **3** in 1:1 $\text{H}_2\text{O}:\text{MeOH}$, neither for **1** dissolved in water. The quality of regressions was visually assessed by analysing the residuals plotted in Figure S8, which are randomly scattered around zero, indicating that the bi-exponential model (eq. 2) fits well to the data. The fitted singlet oxygen lifetimes (τ_{Δ}) are ca. $10 \pm 1 \mu\text{s}$ for the three complexes **1** – **3**, which corresponds to the expected value in air-equilibrated MeOH [69].

Complex **1** was the best singlet oxygen producer with a $^1\text{O}_2$ quantum yield of $\Phi_{\Delta} = 15.0 \pm 1.8\%$. Upon cysteine coordination in complex **2**, a weak $^1\text{O}_2$ signal could be detected ($\Phi_{\Delta} = 1.2 \pm 0.1\%$), indicating that coordination of a thiolate ligand influenced the ability of the $\text{Re}(\text{CO})_3(\text{bpy})^+$ species to generate $^1\text{O}_2$. This is a significant observation, because in a complex biological

environment, the aqua ligand of **1** would be susceptible to substitution with a variety of thiol-containing biomolecules. Complex **3** appeared to be a slightly better $^1\text{O}_2$ generator ($\Phi_{\Delta} = 8.5 \pm 0.9\%$) than **2**, which could be attributed to the formation of *fac*- $[\text{Re}(\text{CO})_3(\text{bpy})(\text{CH}_3\text{OH})]^+ \text{Na}^+$ (NAC^{2-}) in MeOH solution (see eq. 3).

Detection of CO Release

To further investigate the photochemistry of Re-HCys (**2**) and Re-NAC (**3**) complexes, solutions of **2** (12.5 μM) and **3** (20 μM) in deoxygenated methanol were placed in a Schlenk cuvette under argon atmosphere, and then irradiated using a 365 nm UV-A hand lamp, measuring their UV-vis spectra at 20-minute time intervals (see Figure S9(a)). The changes in IL and MLCT bands in the absorption spectra, as well as observation of isosbestic points, would indicate conversion of one species to another through the release of a CO ligand [20]. Although the spectral changes observed for complexes **2** and **3** were minimal, a closer look at these changes after 2 h irradiation shows that their spectral features resemble that of the Re-Aq complex (**1**) shown in Figure 3; see the comparison in Figure S9(b). This could be attributed to the exchange between the Re-bound thiolate ligands and solvent (MeOH) molecules over 2 h photolysis. The release of CO was also measured using gas chromatography with a thermal conductivity detector (GC-TCD). Solutions of **1**, **2** and **3** (50 μM) in deoxygenated MeOH were irradiated using the same apparatus over a period of 2 hours, with minimal to no CO being detected (see Table S6).

Earlier studies on $[\text{Re}(\text{CO})_3(N,N)\text{L}]^{+/0}$ complexes have shown that the nature of the ligand trans to CO ($_{\text{axial}}$), such as phosphine-based ligands, can influence the release of the axially bound CO, due to their *trans*-directing abilities [20, 30]. We used *fac*- $[\text{Re}(\text{bpy})(\text{CO})_3(\text{P}(\text{CH}_2\text{OH})_3)](\text{CF}_3\text{SO}_3)$ as reference, and repeated the same experiment using

degassed PBS as solvent (Figure S10), which led to near identical results as reported in the literature ($\lambda_{\text{irradiation}} = 365 \text{ nm}$; solvent = PBS, pH = 7.4) [30], indicating that the experimental setup and apparatus used were sufficient for monitoring CO release. In the present study, the HCys⁻ and NAC²⁻ ligands were not efficient *trans*-directors, and therefore did not induce CO release.

3.3. Cytotoxicity and Cellular Localization

Cell Viability. Complexes **1** and **2** were chosen for further cell viability assessment, tested against the MDA-MB-231 breast cancer cell line using the alamarBlue assay. Cisplatin was also measured for comparison. The instability of complex **3** in various solvents, and impurity of complex **4** led to no further investigation on these complexes. Experiments were performed at the time points of 24 and 48 h (Figure 5, Table 3). Complex **1** displayed cytotoxicity and an IC₅₀ of $38 \pm 6 \text{ }\mu\text{M}$ over the course of 24 h, which appears to outperform cisplatin that does not show significant cytotoxicity at 24 h. At 48 hours, the IC₅₀ value of **1** dropped to $26 \pm 3 \text{ }\mu\text{M}$, whereas that of cisplatin was $11 \pm 2 \text{ }\mu\text{M}$ (Table 3). An IC₅₀ value of $15 \pm 5 \text{ }\mu\text{M}$ using the HeLa cell line for **1** has been previously reported (over 72 h) [27]. Since complex **1** displayed cytotoxic activity in the dark, its photocytotoxicity was not measured, despite its ability to produce ¹O₂ upon irradiation. An ideal photosensitizer would not be toxic in the dark, rendering applicability of complex **1** as a photosensitizer doubtful. Despite this, its activity as a chemotherapeutic drug is somewhat promising, beside other complexes with the general formula, *fac*-[Re(CO)₃(*N,N*)(H₂O)]⁺ [27].

Figure 5. Dose-response curve of **1**, **2** and cisplatin at 24 h and 48 h. Data are represented with error bars showing their corresponding standard deviations from three independent experiments with 9 replicates. Data were analyzed with a one-way ANOVA, followed by Dunnet post-tests from comparisons between treated and control cells.

Table 3. IC₅₀ Values of **1**, **2** and Cisplatin in MDA-MB-231 Breast Cancer Cells (see Figure 5)

Complex	IC ₅₀ (μM) ^a	
	24 Hours	48 Hours
1	38 ± 6	26 ± 3
2	>100	>100
Cisplatin	>100	11 ± 2

^a Data are represented as IC₅₀ values ± standard deviations from three independent experiments with 9 replicates.

When considering **2**, the replacement of the aqua ligand in **1** with *L*-cysteine appears to have a significant impact on cytotoxicity. At both time points, IC₅₀ values of over 100 μM were recorded for **2**. An earlier study showed that the co-administration of *L*-cysteine with cisplatin reduced the number of apoptotic cells in rats, indicating that it can act as a protecting agent [70]. *N*-acetyl-*L*-cysteine, known for its antioxidant properties, has been shown to have protecting effects against cisplatin-induced toxicities and damages [71-73]. Our results in this study suggest that the formation of a Re-sulfur bond by *L*-cysteine can reduce the cytotoxicity, and therefore, the efficacy of complex **1**.

Cellular Localization

The cellular accumulation of complexes **1** and **2** were assessed and their elemental distribution maps were obtained using X-ray Fluorescence Microscopy (XFM) [74]. For imaging techniques such as confocal or optical fluorescence microscopy, it is essential for the sample to emit visible fluorescence light upon excitation [75]. However, the Φ_F values for complexes **1** and **2** (specially

for **2**) were minimal (Table 2). Moreover, there have been reports of quenching of the luminescence properties of Re organometallic in the cellular environment [76, 77]. Different with confocal or optical fluorescence microscopy, XFM does not rely on luminescence properties of the complexes. Figures 6 and S11(a) show elemental distribution maps of MDA-MB-231 breast cancer cells (**1**-A, **1**-B and **1**-C) treated for 6 h with a 20 μ M solution of **1** in DMEM, with X-ray fluorescence (K_{α} and L_{β}) peaks of different elements shown for a sample cell in Figure S12 (*top*). Rhenium accumulation in the nuclear and/or perinuclear region of these cells is evident. The nuclear region can be identified via morphology in the optical image, as well as by the presence of zinc and phosphorous, which are predominantly found in the nucleus in zinc finger proteins and in the DNA backbone, respectively [46, 78]. When comparing the Zn and Re maps in cells treated with **1**, there appears to be some overlap between the Zn and Re signals in the nuclear regions of **1**-A and **1**-B, indicating that these two elements to some extent co-localize in these cells. Rhenium accumulation mainly occurs in the perinuclear area of **1**-C cell (rather than the nuclear region); see Figure S11(a).

A recent report on cellular accumulation of a Re(I) tricarbonyl tetrazolato complex, *fac*-[Re(CO)₃(phen)L] (phen = 1,10'-phenanthroline; L = 5-(4-iodophenyl)tetraazolate), also revealed using the XFM technique that Re was localized within the diffuse reticular network in the nuclear and/or perinuclear region in 22Rv1 cells, and that the complex remained intact after uptake. Earlier studies had shown that cellular localization of such complexes varied depending on the substitution group on the aryltetrazolate ligand [78].

Another imaging study by Wilson's group, based on confocal fluorescence microscopy, suggested that a potential antitumor agent Re(I) tricarbonyl complex similar to **1**, i.e. *fac*-[Re(CO)₃(dmphen)(H₂O)](CF₃SO₃), was distributed throughout the cytosol of HeLa cells, being

primarily localized in lysosomes and highly accumulated in the outer membranes of cytoplasmic vacuoles [27]. However, the same group recently reported, based on an inductively coupled plasma mass spectrometry (ICP-MS) study on ovarian cancer cells, that this complex accumulates mainly in mitochondria, with a small detectable amount found in nuclei [28]. The inconsistency between the results of these two studies was explained based on the sensitivity and dependency of Re(I) luminescence properties to its speciation, which can be changed upon its interaction with endogenous ligands, thus limiting the effectiveness and accuracy of data from confocal fluorescence microscopy. Our photophysical data in *Section 3.2* provides strong evidence to attest to the above explanation: the fluorescence quantum yield of **1** changed from 0.037 ± 0.004 to 0.0010 ± 0.0002 upon replacing H₂O in **1** with a cysteinate ligand in **2** (Table 2).

The above studies on cellular localization of *fac*-[Re(CO)₃(*N,N*)L]^{0/+} complexes show that the change in diimine (*N,N*) or axial L ligands can alter the extent and/ or the site of cellular accumulation, which is important when targeting cellular organelles other than DNA, offering alternative modes of cell death [14, 79], helping overcome issues such as platinum resistance mechanisms [80]. It also highlights the importance of using alternate techniques, such as synchrotron-based XFM, to ascertain the cellular accumulation of metal-based drugs. In the present study, it is particularly difficult to pinpoint the exact position of complex **1** in the cells in Figures 6 and S9(a), other than being present in the nuclear and/or perinuclear region, as the distribution images themselves are two-dimensional projection of three dimensional objects in dried cells [78]. This also applies to quantification of elemental concentrations shown in Figure 7, which includes the area both over and under the nucleus such as endoplasmic reticulum [78].

Figure 6. Optical micrographs (*top left*), and XRF elemental distribution map of MDA-MB-231 cells treated for 6 h with **1** (cell **1-A**), **2** (cell **2-A**) and DMEM as a control (**Control-A**). The maximum elemental area densities (quantified from standards and expressed in $\mu\text{g cm}^{-2}$) are given in the bottom of each map.

Figure 7. Intracellular content of Re, P, Zn, Ca, Cu, S, K, Cl and Fe obtained by quantification using XFM as compared with the nuclear content of MDA-MB-231 cells treated for 6 h with control (green, number of cells tested with XFM: $n = 5$) as well as 20 μ M solutions of **1** in DMEM (blue, $n = 4$) and **2** in 1.5% EtOH in DMEM (orange, $n = 5$). Error bars represent standard deviations. Data were analyzed with a one-way ANOVA followed by Tukey post-tests: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ from comparisons between treated and control cell/nucleus regions.

When looking at intracellular content of different elements in all cells treated with **1**, an increase in K and Cl ions can be seen when compared to cells treated with DMEM, or **2** (Figure 7). A possible explanation for such increase is the use of PBS during sample preparation, thus exposing cells to higher concentrations of K and Cl ions. We also observed a significant decrease in copper accumulation in cells treated with **1** and **2**, with respect to control. This observation is independent of the cytotoxicity results.

When considering the cytotoxicity and IC₅₀ value of **2**, which was significantly reduced in comparison to **1** (Table 3), the elemental distribution maps offer an explanation. In Figures 6 and S11 (b), the Re signal in cells treated with **2** is significantly low, with no strong overlap with Zn in the nuclear region. For example, maximum elemental densities of Re in cells **1**-A and **2**-A in Figure 6 are 0.127 and 0.0125 $\mu\text{g cm}^{-2}$, respectively. The quantification histograms (Figure 7) show increased amounts of Re in the cells treated with complex **1** (number of cells tested with XFM: n = 4) as compared to complex **2**, which has little to no Re in a range of cells treated (n = 5). This difference may indicate that the replacement of the aqua ligand in **1** with cysteinate (in **2**) hinders its cellular accumulation and the passage through the cell membrane, a phenomenon that could be related to their charge difference (**1** has positive charge, and **2** is neutral). An earlier study on a series of Re(I) tricarbonyl indolato complexes with strong light-induced antiproliferative activity emphasized on the importance of their neutral charge and membrane localization on their photocytotoxic effect [21]. In the current study, although the Re-HCys complex (**2**) is neutral, its cellular accumulation is very limited (IC₅₀ > 100 μM).

Chemotherapeutic drugs are typically administered intravenously, resulting in exposure to blood plasma and a variety of amino acids and biomolecules, which may interact with the drug

before it reaches the cellular environment [81-83]. This highlights the importance of studies (such as this one) focusing on interactions between potential anticancer agents and biomolecules, and how such interactions would affect their activity and cellular accumulation.

4. CONCLUSION

This study reports the syntheses of two complexes formed from the reaction of a known anticancer active complex, *fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) (**1**), with thiol-containing biomolecules, i.e. the amino acid *L*-cysteine, the tripeptide glutathione, and *N*-acetyl-*L*-cysteine, which is a potent antioxidant with clinical applications. The crystal structure of {*fac*-[Re(CO)₃(bpy)(HCys)]}₄·9H₂O (**2** + 1.75 H₂O) is also reported. Rhenium L₃-edge EXAFS spectroscopy confirmed a similar local structure for Re(I) ions in its glutathione and *N*-acetylcysteine complexes in solid state. Complexes with cysteine and *N*-acetylcysteine were insoluble in water, while the Re-glutathione complex easily dissolved in water at physiological pH, making its purification process quite challenging. Cell viability experiments against the MDA-MB-231 breast cancer cell line using **1**, **2** and cisplatin showed **1** had a significant increase in cytotoxicity over the course of 24 h. When measured again at a 48 h time point, the activity of cisplatin had surpassed that of **1** possibly indicating a different mechanism of action between the two complexes. At both time points, the cytotoxic activity of **2** (dissolved in 1.5% EtOH) was significantly reduced, highlighting the effect of replacing the aqua ligand in **1** with *L*-cysteine. The cellular localization was monitored using synchrotron-based X-ray fluorescence microscopy (XFM), which showed **1** accumulated in the nuclear and/or perinuclear regions, partially co-localizing with Zn, while the accumulation of **2** into the cell was considerably lower and may be hindered, explaining its reduced cytotoxic activity.

The photophysical properties of complexes **1**, **2** and Na[Re(CO)₃(bpy)(NAC)] (**3**) were also assessed in methanol. The excitation and emission spectra displayed a large variance in Stokes shift between all three complexes. The major change in the emission spectra of **1** and the Re-cysteine complex **2** highlights the impact of complex formation with biomolecules on emission properties and visibility by confocal / fluorescence microscopy. The generation of ¹O₂ in MeOH was observed for complexes **1** and **3**, but not for **2**. This again indicates that the coordination of cysteine to [Re(CO)₃(bpy)]⁺ entity deactivates ¹O₂ generation. The release of CO upon irradiation with UV-A light ($\lambda = 365$ nm) was not observed for any of the complexes, showing that these thiol-containing biomolecules or the aqua ligand are poor *trans*-directors for activating their trans CO group, and therefore, their Re(I) diimine tricarbonyl complexes would not be efficient as a PACT agent.

ASSOCIATED CONTENT

Electronic Supplementary Materials. The online version of this article contains supplementary material, which is available to authorized users.

Accession Codes

CCDC 1981028 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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ABBREVIATIONS

ESI-MS, electrospray ionization mass spectrometry; EXAFS, extended X-ray absorption fine structure spectroscopy; XFM, X-ray fluorescence microscopy; PDT, photodynamic therapy; PACT, photo-activated chemotherapy; MT, metallothionein; GC-TCD, gas chromatography with thermal conductivity detection; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; TD-DFT, time-dependent density functional theory; MLLCT, metal-ligand to ligand charge transfer; ILCT, intra-ligand charge transfer; bpy, 2,2'-bipyridine; phen, 1,10-phenanthroline; dmphen, 2,9-dimethyl-1,10-phenanthroline; DMEM, Dulbecco's Modified Eagles Medium; PBS, phosphate buffered saline; IC₅₀, half-maximal inhibitory concentration.

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Graphical Abstract

Replacing the aqua ligand with cysteine in the antitumor active *fac*-[Re(CO)₃(bpy)(H₂O)](CF₃SO₃) complex significantly reduced its cellular accumulation and cytotoxicity against the MDA-MB-213 breast cancer cell line, shifted its maximum emission to considerably higher energies, and decreased its fluorescence quantum yield.