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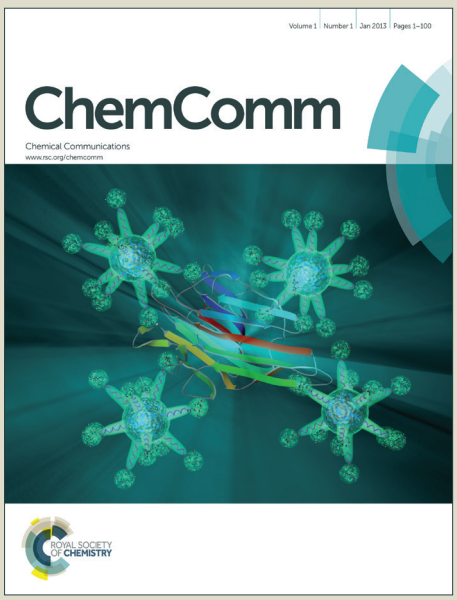

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ARTICLE TYPE

Receptor Selective Ruthenium-Somatostatin Photosensitizer for Cancer Targeted Photodynamic Applications

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The efficient conjugation of a Ruthenium complex and the peptide hormone somatostatin is presented. The resultant biohybrid offers valuable features for photodynamic therapy such as remarkable cellular selectivity, rapid cell uptake by receptor-mediated endocytosis, efficient generation of $^1O_2$ upon irradiation, potent phototoxicity as well as low cytotoxicity in the "off"-state.

Photodynamic therapy (PDT) is a non-invasive modality for the treatment of various types of cancers (*e.g.* lung, esophagus, skin tumors).[1] By utilizing light irradiation, PDT activates nontoxic photosensitizers (PS) to generate radicals or singlet oxygen ($^1O_2$) species, which initiates apoptosis and/or necrosis and eventually leads to cell death.[2] Spatiotemporal control over the release of the active species has resulted in selective destruction of tumor cells within the irradiated area, potentially reducing the dose-limiting side effects incurred with conventional chemotherapy.[3, 4]However, the classical PS usually lack sufficient selectivity for tumor cells and cause collateral damage to surrounding healthy cells.[5] Therefore, it is highly desirable to incorporate tumor-specific targeting moieties onto PS for dual selectivity, allowing preferential accumulation of PS in tumor cells while providing spatial irradiation control of the tumor site.[6] Various tumor targeting molecules have been investigated *e.g.* antibodies, folic acid, transferrin, RGD as well as aptamers.[7, 8] Among them, tumor-specific peptides offer many attractive features such as non-immunogenicity, high tissue penetration, high affinity to cellular biomarkers as well as straight forward conjugation chemistry.[9] Although the coupling of a tumor-specific peptide to photosensitizers such as porphyrins has been well studied,[10, 11] the conjugation of Ruthenium complexes with tumor-targeting peptides for targeted PDT has not been described yet.

Conventional porphyrin-based PDT agents reveal a number of the following limitations, such as hydrophobicity, poor light absorption, lack of specificity, dark toxicity and prolonged skin sensitivity.[12] Instead, the anticancer activity of Ruthenium(II) complexes has been extensively investigated since they combine many attractive features for PDT such as favorable photophysical properties, facile synthesis, tunable physical properties (*i.e.* charge, lipophilicity or redox potential by coordination of the appropriate ligands), insensitivity of photochemical properties to pH-value variations and low toxicity toward healthy tissues.[13] Ruthenium(II) complexes have demonstrated great potential for PDT,[14] and one of them, TLD-1433 from the group of McFarland, will enter phase I clinical trials.[15] Therefore, Ruthenium(II) complexes and their bioconjugates are particularly attractive for targeted PDT. However, the application of the commonly used Ruthenium polypyridyl complexes in PDT is still restricted by their low cell uptake and poor cell type selectivity.[16]To the best of our knowledge, Ruthenium conjugates offering dual selectivity, *i.e.* selective accumulation into tumor cells or tissue *via* receptor-mediated cell uptake *via* membrane receptors that are overexpressed within the membrane of certain tumor cells and spatially selective photoactivation to induce cellular toxicity only at the tumor site are still elusive.[17-19] The development of Ruthenium conjugates with such "dual selectivity" would be highly attractive for cancer therapy.

Somatostatin receptors (SSTRs), especially SSTR subtype 2 (SSTR 2) offers tumor cell and neovasculature targeting since they are overexpressed in various tumor cells and in tumor blood vessels relative to normal tissues.[20] The endogenous peptide hormone somatostatin (SST) exerts its biological effects through SSTRs with high binding affinity in the nanomolarrange.[20] Therefore, SST and its analogues have been widely investigated for targeted drug delivery into SSTR-expressing cancer cells by bioconjugation to radionuclides and antineoplastic agents.[21]

For the conjugation of SST, commonly used approaches *via* non-specific lysine modification are not applicable as a lysine residue is located within the SST receptor binding domain. To retain the binding properties of the SST, N-terminal modification *via* solid phase synthesis has been applied successfully although tedious synthesis, exhaustive purification and low overall yields represent significant limitation. Herein, the modification of the single disulphide bond of SST offers a facile synthetic strategy to access defined SST conjugates with conservedbioactivity.[22, 23]

Bis-alkylation reagents have been developed for peptide or protein modification *via* disulfide rebridging.[24] They rebridge the solvent accessible disulfide bonds of peptide or proteins *via* two consecutive Michael addition reactions without the loss of their biological activity or structural integrity.[25, 26] This strategy has been successfully applied for the modification of peptide hormones,[23] antibodies,[26, 27] and therapeutic proteins.[25] In addition, disulfide bonds in peptides and proteins are often unstable under physiological conditions due to disulfide scrambling. Therefore, the disulfide rebridging provides thioether conjugates with improved stability.[22] It also renders the

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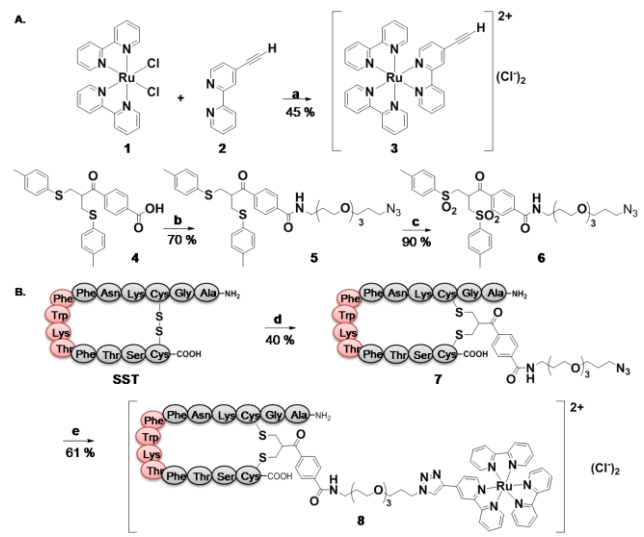
conjugates responsive to intracellular glutathione and dissociation occurs in the presence of elevated tumor GSH concentrations thus providing a valuable strategy to control anti-tumor drug release in the cytosol of cancer cells.[22]

Herein, we have designed Ru-SST **8** with attractive phototoxicity and tumor cell selectivity by combining the unique features of the peptide hormone SST and the PS $[Ru(bpy)_3]^{2+}$. For instance, potent SSTR2 agonistic activity facilitates transport into SSTR-positive cells for tumor cell specific drug delivery[22] and controlled photoactivation of the nontoxic prodrug $[Ru(bpy)_3]^{2+}$ induces cytotoxicity only after irradiation in a spatially and temporally controlled fashion.[28] Thus, Ru-SST offers many important features for expanding the anti-tumor features of Ru-complexes such as attractive receptor selective cellular uptake, light-controllable cytotoxicity, potent anti-proliferative effects and low systemic toxicity.



Scheme 1. A. Synthesis of the disulfide rebridging and bioconjugation reagent **6.** a. ethanol/water 3:1, reflux 3 h; b. HBTU, DIEA, DMF, overnight; c. Oxone, MeOH/H_2O 1:1, 24 h. B. Functionalization of SST to receive Ru-SST conjugate **8.** d. 2 eq. TCEP for 0.5 h, then compound **6** for 24 h, 50 mM phosphate buffer pH 7.8; e. 2 eq. $CuSO_4$, 4 eq. Na ascorbate, H_2O, overnight.

A detailed synthetic route for Ru-SST **8** is shown in Scheme 1. Ru-Alkyne **3** was obtained by refluxing $(bpy)_2RuCl_2$ **1** and bpy-Alkyne **2** in ethanol/water 3:1 for 3 h, followed by sephadex column chromatography purification affording Ru-Alkyne **3** in 45% yield. Compared to other synthetic methods, this approach proceeds without the addition of cytotoxic Ag^+ ions, which is detrimental for biomedical applications in living systems.[3, 29, 30] Subsequently, Ru-Alkyne **3** was conjugated to SST *via* extremely efficient Cu(I) catalysed cycloaddition in water with full conversion of SST (Scheme 1). Ru-SST conjugate **8** was isolated in 61% yield after HPLC purification and characterized by HR-ESI-MS (Fig. S1), HR-MALDI-MS (Fig.S2) and LC-ESI-MS (Fig. 1A). The isotopic patterns of the peaks found in HR-MS correspond with the theoretical calculations (Fig.S1-2). Noteworthy, Ru-SST **8** was observed only as singly charged $[M-1]^+$ species in HR-MALDI-MS measurements indicating a reduction during the MS measurement. This finding is in accordance with previous reports in the literature.[31]

The UV/vis absorption and photoluminescence spectra have been recorded in MilliQ water (Fig.1C and 1D). Both Ru-Alkyne **3** and Ru-SST **8** reveal typical photophysical behaviour of Ruthenium(II) polypyridine complexes (Table S1-2).[32] The most relevant absorption transition, the metal-to-ligand-charge-transfer (MLCT) of Ru-SST **8** (458 nm) is identical to **3** (457 nm). Furthermore, additional characteristic absorption transitions both for Ru-SST **8** and **3** are presented in Table S1. The phosphorescence-type emission is observed at 621 nm for Ru-SST **8** and 631 nm for **3**. The hypsochromic shift of the emission wavelength correlates to the altered nature of the substituent *i.e.* the change from alkyne to triazole. For Ru-SST **8**, a significantly increased luminescence intensity compared to Ru-Alkyne **3** is observed, which could tentatively be explained by enhanced non-radiative deactivation as the MLCT state in **3** is lower in energy.[33]

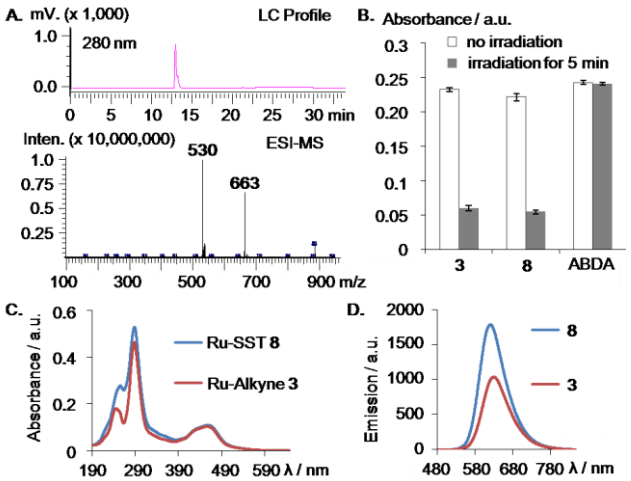


Fig. 1. Characterization of Ru-SST **8.** A. LC-MS spectra of Ru-SST **8**, m/z= 530 $[M^{5+}]$, 663 $[M^{4+}]$. (calcd. exact mass: 530.61486 $[M^{2+}+3H]^{5+}$, 663.01670 $[M^{2+}+2H]^{4+}$, formula: $C_{129}H_{158}N_{28}O_{24}RuS_2$).B. UV-vis absorption spectra for the photobleaching of ABDA(20 μM) during the irradiation of Ru-Alkyne **3** and Ru-SST **8** (10 μM) in PBS (1x, pH7.4) over a period of 5 min. C. The absorbance spectra of Ru-SST **8** in H_2O. D. The emission spectra of Ru-SST **8** in H_2O (λ_{ex} = 460 nm).

As some Ruthenium(II) polypyridine complexes are known to be photolabile, their photostability was examined by monitoring the absorption after irradiation with visible LED light (λ = 470 nm, P = 50 ± 3 mW, 50± 3 mW/cm^2).[34] Ru-SST **8** and Ru-Alkyne **3** retained all their characteristic absorption bands with a slight decay of 8 % and 7%, respectively. This indicates a significant improvement in photostability compared to $[Ru(bpy)_3]Cl_2$, which featured a decay of 21% under analogous conditions (Fig.S3).

Importantly, the consequent application of PDT relies on the production of singlet oxygen. The generation of $^1O_2$ of **3** and Ru-SST **8** was investigated by applying two different methods. First, emission quenching caused by oxygen was examined by monitoring the emission spectra in both oxygen free and oxygen saturated MilliQ water (Fig.S4). An emission decay of 35% for Ru-SST **8** and 29 % for Ru-Alkyne **3** was detected. These quenching values correspond well to the related $[Ru(bpy)_3]Cl_2$ with a quenching value of 41 % in water.[35] This effect is the result of an energy transfer from the electronically excited Ruthenium(II) complex in its ^{3}MLCT state to oxygen in its ground state ($^3O_2$) potentially forming electronically excited singlet oxygen ($^1O_2$), which is a reactive oxygen species (ROS).[36]

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In order to verify the generation of $^1O_2$, the singlet oxygen sensor 9,10-anthracenediyl-bi(methylene) dimalonic acid(ABDA) was applied. In the presence of $^1O_2$, an endoperoxide of ABDA is formed, which decreases the ABDA absorption at 380 nm thus providing a valuable means of direct monitoring $^1O_2$ production. Ru-Alkyne **3** and Ru-SST **8** (10 μM) were mixed with 20 μM of ABDA in PBS buffer and then irradiated by a 470 nm LED array with P = 50 ± 3 mW for 5 min (15 ± 0.9 J/cm^2). As shown in Fig.1B, the generation of singlet oxygen by Ru-Alkyne **3** and Ru-SST **8** resulted in more than 70% decrease in ABDA absorption at 380 nm, indicating efficient $^1O_2$ generation.

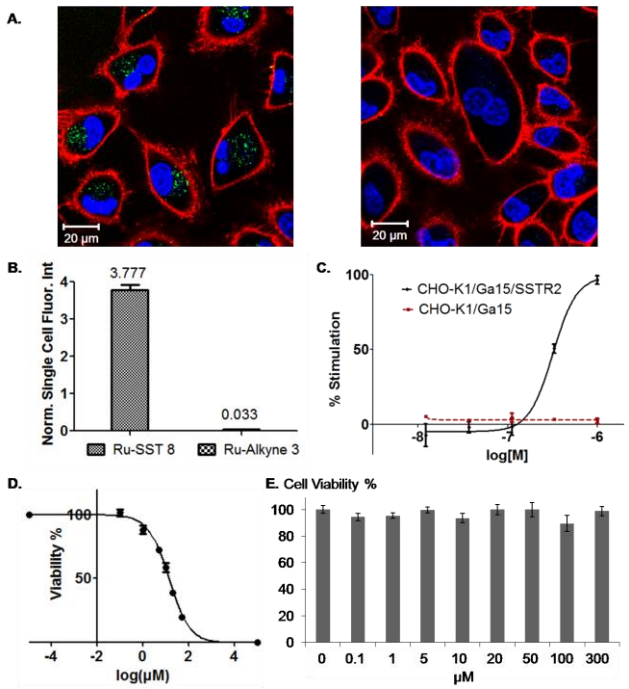

Fig. 2. A. Confocal microscopy images of 10 μM Ru-SST **8** (left) and Ru-Alkyne **3** (right) incubated with A549 cells for 4 h. B. The normalized single cell emission intensity (Single cell emission intensity/area of cell) was quantified by ImageJ software based on the confocal images (Fig.S6). The normalized single-cell emission intensity with the incubation of Ru-SST **8** was divided by 1.83, since the Ru-SST **8** has 1.83 times higher emission than Ru-Alkyne **3**. Data are plotted as means ± standard errors of the means (SEM) using GraphPadPrism5 Software (n=15).C. Calcium flux induced by Ru-SST **8** (EC_{50} = 319.6 **±** 1.1nM) in SSTR2 expressing CHO-K1 cells (black) and wild type CHO-K1 cells (red). D. Cytotoxicity of Ru-SST **8** on A549 cells with light irradiation for 5 min (IC_{50}= 13.2 **±** 1.1μM).E. Cytotoxicity of Ru-SST **8** on A549 cells in the absence of light.

The cellular uptake of **8** into human non-small-cell lung cancer (NSCLC) A549 cells was investigated, since these cells express SSTR subtypes 1, 2, 4 and5 on the cellular surface.[37] Equal quantities of Ru-Alkyne **3** and Ru-SST **8** (10 μM) were added to the culture medium of A549 cells, respectively. After incubation for 4 h, the cells were washed to remove any conjugates that were not taken up and the cells were studied by laser scanning confocal microscopy. Laser excitation at 458 nm was applied that corresponds to the MLCT absorbance of the metal complex. The emission images were recorded in the range of 580-707 nm. As shown in Fig.2A, only minimal emission was observed within the cells after incubation with Ru-Alkyne **3**, while Ru-SST **8** was transported rapidly and efficiently across the membrane and accumulated inside the cells. The emission intensity inside the cells was quantified by the normalized single-cell emission intensity (single-cell emission intensity/area of cell) by using the ImageJ software (Fig.2B). More than a hundred times higher uptake was found compared to the control experiment, indicating significant improvement of cellular transport after conjugation with SST. The efficient cellular uptake was also detected by emission imaging (Fig.S5). Cell type selectivity was demonstrated by applying Ru-SST **8** on wild type CHO-K1/Ga15 cells and CHO-K1/Ga15/SSTR2 cells overexpressing SSTR2 for a functional calcium flux assay conducted by GenScript. Ru-SST **8** revealed significant receptor activation and calcium release already at low concentrations (EC_{50} of 319.6 **±** 1.1 nM) whereas there was no sign of activation on wild type CHO-K1/Ga15 cells, indicating high cell selectivity of Ru-SST towards SSTR2 cells (Fig.2C). To determine the effectiveness of **8** as PDT agents in cancer cells, its photocytotoxic properties were examined on the A549 cell line. The dark and light cytotoxicity profiles for Ru-SST **8** and Ru-Alkyne **3** in A549 cells were screened at a wide range of concentrations (from 0 to 300 μM) to determine their potency (IC_{50}). Briefly, the cells were incubated with Ru-SST **8** or Ru-Alkyne **3** in the dark at different concentrations (from 0 to 300 μM) for 4 h. Subsequently, the cells were washed and irradiated by a 470 nm LED array with P = 23 ± 3 mW for 5 min (6.9 ± 0.9 J/cm^2). The dark controls were performed in parallel. The cells were further incubated for 6 h in the dark. The cell viability was quantified by the Tox-8 assay (Sigma Aldrich) according to the manufacturer's instructions.[38] The Ru-SST **8**induced pronounced cytotoxicity with IC_{50} value of 13.2 **±** 1.1μM (Fig.2D) after light irradiation, while it remained nontoxic up to 300 μM (Fig.2E) in the absence of light. Therefore, Ru-SST **8** displays a high phototoxic index (PI) of greater than 23 (PI is the ratio of the dark and light IC_{50} values), making it very potent at very short drug-to light intervals (4 h) and low dosage of visible light (6.9 ± 0.9 J/cm^2). In addition, Ru-SST **8** (IC_{50}= 13.2 **±** 1.1 μM) elicits superior phototoxicity compared to the Ru-Alkyne complex **3** (IC_{50}= 67.5 **±** 1.1 μM), underlining theimportance ofincreased cellular uptake and thus the greater potential of Ru-SST **8** for PDT (Fig. S7). While existing Ruthenium-peptide conjugates have been exploited for their anticancer activity,[18, 39] transmembrane transport and nuclear targeting,[16, 40] as well as photocontrolled DNA binding,[41] we demonstrate herein for the first time the potent phototoxicity of the receptor-targeted Ruthenium heteroconjugate. Our results clearly demonstrate the great potential of Ru-SST for targeted PDT due to selective targeting of SST positive cancer cells and high phototoxicity while maintaining low systemic toxicity.

Conclusions

We have reported the synthesis, photophysics, cellular uptake and phototoxicity of the first photostable cancer cell type selective Ruthenium(II) polypyridine-SST conjugate Ru-SST **8** with great potential in targeted photodynamic therapy. The functionalization of SST *via* disulfide rebridging provides a facile approach to access defined conjugates with retained activity, compared to solid phase synthesis. The synthesis route gave high yields under mild reaction conditions thus offering a convenient strategy to access even compound libraries of structurally diverse photodynamic agents, in which the bioactive functionalities are

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integrated without compromising their intrinsic properties. The resulting conjugate displayed enhanced luminescence and photostability compared to $Ru(bpy)_3^{2+}$, which is attractive for biological studies. In addition, conjugation of SST initiates cellular uptake *via* receptor mediated endocytosis. Ru-SST **8** was taken up into the SSTR expressing cells with 100-fold increased efficiency compared to Ru-Alkyne **3**. Upon light irradiation, Ru-SST **8** exhibited potent cellular toxicity in the low micromolar range at very short drug-to-light intervals and low dosage of visible light. To the best of our knowledge, we have reported the first Ruthenium-peptide conjugate providing dual selectivity, by combining selective uptake in specific cancer cells and restriction of photoactivation to the tumor site. The efficient conjugation strategy established in this paper will be valuable to further fine-tune the Ruthenium(II) complexing ligands to access photoactivation wavelengths in the typical therapeutic window (600-1000 nm) for targeted PDT.[42-44] Alternatively, two photon absorption concepts or the use of analogous Osmium PS represent emerging concepts towards this aim.[45-47]

Notes and references

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† Electronic Supplementary Information (ESI) available: Synthesis protocols, characterization and cell studies. See DOI: 10.1039/b000000x/

‡ Equal contribution.

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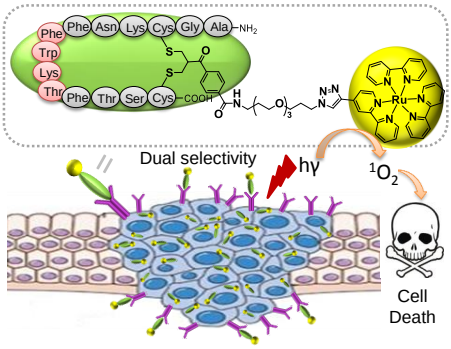

Ru-SST reveals enhanced selectivity for tumor cells due to cellular uptake *via* receptor mediated endocytosis. Upon light irradiation, the localized conjugate generates an immediate burst of reactive oxygen species (ROS), which is restricted to the tumor site, resulting in efficient photo-induced cellular toxicity while potentially avoiding collateral damage to healthy cells.