Bioconjugate Chemistry

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Regulation of Target Protein Knockdown and Labeling Using Ligand-Directed Ru(bpy)$_3$ Photocatalyst

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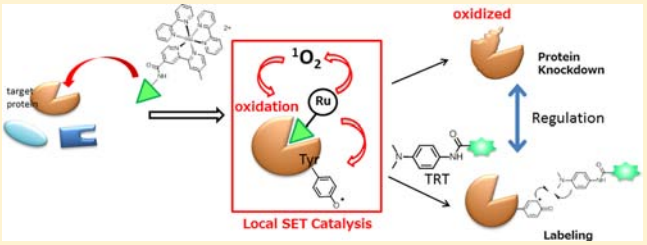
Supporting Information

ABSTRACT: Ligand-directed Ru(bpy)$_3$ photocatalysts induce chromophore-assisted light inactivation (CALI) of target proteins under visible light irradiation in vitro and within cells. Here, histidine, methionine, and tryptophan residues were oxidized by the singlet oxygen ($^1O_2$) generated by Ru(bpy)$_3$ with light. The addition of a tyrosyl radical trapper (TRT), such as *N*′-acyl-*N*,*N*-dimethyl phenylenediamine, inhibited peptide/protein oxidation and induced labeling on the tyrosine residue. This mechanistic study suggests that TRT scavenges $^1O_2$, concomitant with the coupling reaction to the tyrosyl radical generated by Ru(bpy)$_3$. Both CALI and labeling can be regulated by the Ru(bpy)$_3$ photocatalysts in the absence or presence of TRT. Ligand-conjugated Ru(bpy)$_3$ photocatalysts (local environmental single-electron transfer catalysts: LSCs) were used not only for target-selective protein labeling, but also for protein knockdown by CALI.



INTRODUCTION

The inactivation of a protein of interest in cells is a useful method not only for clarifying biological functions, but also for developing therapeutic strategies for specific diseases. In this context, gene silencing technologies using RNA interference have already contributed significantly to life science research.[1] Although these well-established genetic engineering approaches are powerful and versatile, their efficiency depends on the half-life of the target protein in cells, and it might be difficult to effectively knock down proteins with long half-lives.[2] To overcome the inadequacies of these genetic engineering methods, the inactivation of the target protein without gene manipulation has been investigated as an alternative strategy for target protein knockdown.[3]

The chromophore-assisted light inactivation (CALI) of proteins, a protein knockdown technology triggered by light irradiation, is one such potentially powerful tool.[4] Various ligand-directed photocatalysts have been used for target-selective CALI.[5] Kodadek and co-workers developed the technique of selective light inactivation of proteins with peptoid-ruthenium conjugates that targeted intracellular proteins.[6] They used ruthenium(II) tris(bipyridyl) (Ru(bpy)$_3$) as a cell-permeating[7] photocatalyst to generate $^1O_2$.[8]

In contrast, we recently reported single-electron transfer (SET)-based tyrosine residue labeling using Ru(bpy)$_3$ photocatalysts. In this system, a Ru(bpy)$_3$ derivative was used as the SET catalyst to generate tyrosyl radicals, in another function additional to $^1O_2$ generation. We developed synthetic affinity-ligand-conjugated Ru(bpy)$_3$ complexes as local environmental SET catalysts (LSCs), and demonstrated target-protein-selective labeling using a benzenesulfonamide-conjugated Ru(bpy)$_3$ complex targeting carbonic anhydrase (CA) in mouse erythrocytes.[9] The tyrosyl radicals generated by LSC reacted with *N*′-acyl-*N*,*N*-dimethyl phenylenediamines, called "tyrosyl radical trappers" (TRTs), resulting in target-protein-selective labeling in mouse erythrocytes.

In this paper, Ru(bpy)$_3$ was used as a bifunctional photocatalyst: (1) an efficient $^1O_2$ generator[10] for oxidative protein knockdown; and (2) a tyrosyl radical generator[11] for protein labeling. Although the mechanism of $^1O_2$-mediated CALI[12] and the chemical reactions of the free amino acid with $^1O_2$ have been reported,[13,14] the oxidative effects of Ru(bpy)$_3$-mediated protein knockdown on the amino acid residues have not been determined. To clarify the mechanisms of both the protein knockdown and the labeling induced by ligand-directed Ru(bpy)$_3$, we mechanistically studied the effects of TRT on the regulation of protein knockdown and labeling using a peptide and the model target protein CA. We found that TRT is essential for the regulation of both reactions in cells. Protein knockdown is promoted by LSC alone, whereas the labeling reaction is promoted by LSC and TRT. This is the first example of the regulation of both protein inactivation and labeling by a single photocatalyst.

RESULTS AND DISCUSSION

Regulation of Peptide Oxidation and Labeling. We first examined the effect of the light-activated Ru(bpy)$_3$ complex on the oxidation of various peptides using mass

Received: November 11, 2014
Revised: December 6, 2014
Published: December 30, 2014

DOI: 10.1021/bc500518t
Bioconjugate Chem. 2015, 26, 250−256

spectrometry (MS). The light irradiation of angiotensin II (DRVYIHPF) with $Ru(bpy)_3$ resulted in the complex oxidation of angiotensin II (Supporting Information (SI) Figure S1). The MS peak of oxidized angiotensin II disappeared in matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis. In contrast, the mass peaks of bradykinin (RPPGFSPFR) and an angiotensin II variant (DRVYIAPF), which contain no histidine residues, were unaffected under the same conditions (SI Figures S2 and S3). Liquid chromatography (LC)-MS analysis of the oxidized angiotensin II suggested that the histidine residue was oxidized by the $^1O_2$ generated by $Ru(bpy)_3$, resulting in a complex mixture of various oxidized forms (SI Figure S4).[14] We also examined the effects of the light-activated $Ru(bpy)_3$ complex on other oxidative-stress-sensitive amino acid residues, including cysteine, methionine, and tryptophan (SI Figures S5–S8). The mass peaks of the peptide containing methionine and tryptophan were shifted to the oxidized form: Met + O (+16 Da) and Trp + O_2 (+32 Da), respectively. However, the tyrosine and cysteine residues were not oxidized by light-activated $Ru(bpy)_3$.

In the presence of TRT (**1**) (Figure 1), the tyrosine residue of angiotensin II was labeled with **1** and oxidation was barely

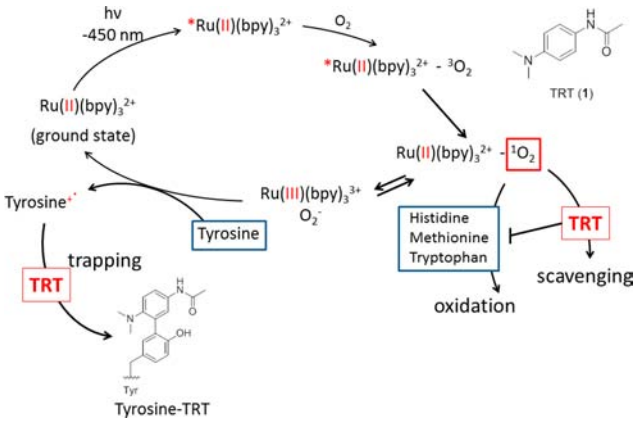


Figure 1. Proposed mechanism of $^1O_2$ generation and tyrosine residue labeling.

detectable (SI Figure S9). We found that the addition of **1** suppressed the oxidation of histidine, methionine, and tryptophan induced by the $^1O_2$ generated (SI Figures S10–S12). These results indicate that $Ru(bpy)_3$-mediated peptide oxidation and tyrosine labeling are regulated by the addition of TRT.

Mechanistic Studies. To clarify the mechanism underlying the suppression of oxidation by TRT, the effect of TRT on $^1O_2$ was measured. Singlet oxygen sensor green (SOSG) was used to detect $^1O_2$ (SI Figure S13).[15] The light-activated $Ru(bpy)_3$ complex and a $^1O_2$-generating reagent, 3-(1,4-epidioxy-4-methyl-1,4-dihydro-1-naphthyl)propionic acid (EP),[16] induced an increase in SOSG fluorescence intensity. The addition of **1** inhibited the increase in SOSG fluorescence intensity caused by both light-activated $Ru(bpy)_3$ and EP (SI Figures S13 and S14). These results suggest that TRT acts not only as a tyrosine-labeling agent, but also as a mild $^1O_2$ scavenger. Highly concentrated sodium azide (NaN_3), a $^1O_2$ scavenger, strongly inhibited the TRT labeling of angiotensin II catalyzed by the light-activated $Ru(bpy)_3$ complex (SI Figure S15). However, the $^1O_2$ generated by EP did not induce the TRT labeling of angiotensin II (SI Figure S16). These results indicate that the tyrosine-labeling reaction proceeds via the generation of $^1O_2$, and $^1O_2$ probably contributes to the generation of the Ru(III) state required for tyrosine residue labeling. Addition of superoxide dismutase accelerated the TRT labeling, strongly suggesting the contribution of Ru(III) state and the existence of equilibrium state between Ru(II) and Ru(III) (SI Figure S17).

Together with data from our previous mechanistic study of TRT labeling,[9] the present data were used to construct an overview of the proposed mechanism, shown in Figure 1. The energy transfer from light-activated Ru(II) to molecular oxygen provides $^1O_2$. Histidine, methionine, and tryptophan residues are oxidized by $^1O_2$ in the absence of TRT. SET from light-activated Ru(II) to $^1O_2$ results in the generation of Ru(III) and a superoxide anion radical (O_2^{-}), and SET from a tyrosine residue to Ru(III) generates a tyrosyl radical. Thus, TRT plays two roles: as a $^1O_2$ scavenger and a tyrosyl radical trapper.

Regulation of Carbonic Anhydrase Knockdown and Labeling. Because peptide oxidation and labeling can be regulated by TRT, we next examined target-protein-selective CALI and labeling using LSCs under light irradiation. We chose benzenesulfonamide (**2**) as the ligand of bovine carbonic anhydrase II (CA), and demonstrated CA-selective CALI and labeling using the benzenesulfonamide-conjugated $Ru(bpy)_3$ complex, Ru-SO_2NH_2 (**3**), as the LSC and biotin-TRT (**4**) in a 1:1 mixture of bovine serum albumin (BSA) and CA. In the absence of **4**, **3** induced CA-selective oxidation (Figure 2, lane

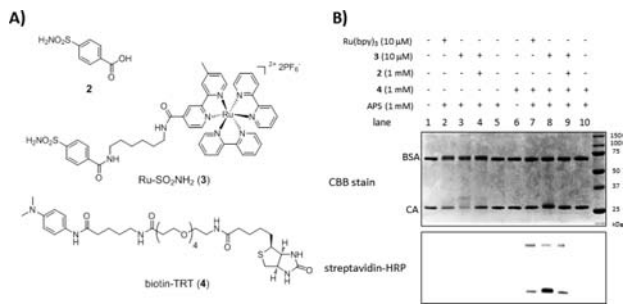


Figure 2. Regulation of CA-selective knockdown and labeling by the addition of TRT. (A) Chemical structures of the CA ligand (**2**), CA-ligand-conjugated $Ru(bpy)_3$ (**3**), and biotin-conjugated TRT (**4**). (B) CA knockdown and labeling were performed in the absence and presence of **4**. The reactions were performed under light irradiation for 30 min.

3). The oxidized CA showed "smearing" in the gel because the different oxidized forms of the protein differ in their electrophoretic mobility, as reported previously.[17] The Ru-$(bpy)_3$ complex, which cannot interact with CA, did not induce CA oxidation (lane 2). The addition of an excess amount of competitor **2** inhibited CA oxidation (lane 4). The additive oxidant, ammonium persulfate (APS), did not induce "smearing" of the proteins (lane 1 vs lane 5). APS was added to enhance the $^1O_2$-generation induced by light-activated $Ru(bpy)_3$ (see SI Figure S18). In the presence of biotin-TRT **4** (lanes 6–10), the $Ru(bpy)_3$ complex labeled both BSA and CA (lane 7), whereas when **3** was used, CA was selectively labeled (lane 8). CA-selective labeling was inhibited in the presence of competitor **2** (lane 9). It must be noted that CA oxidation was inhibited in the presence of biotin-TRT **4** (lanes 6–10).

DOI: 10.1021/bc500518t
Bioconjugate Chem. 2015, 26, 250–256

Mechanism of Carbonic Anhydrase Knockdown. We then examined whether CA-targeting CALI inactivated its actual enzymatic function. CA was treated with **3** in the dark or under light irradiation. Although part of the enzymatic activity of CA was inhibited by **3** without light irradiation, probably because **3** bound to the active site of CA, the inhibitory activity of **3** was enhanced by light irradiation (SI Figure S19). To determine the oxidized regions in CA, CA oxidized with light-activated **3** was digested in an SDS-PAGE gel, and analyzed with MALDI-TOF MS. When the digested fragment peaks of intact CA were observed, two regions of amino acid sequence, amino acids 2−18 (2−9 and 10−18) and amino acids 227−251, were not observed in the oxidized CA, suggesting that these regions were selectively oxidized with light-activated **3** (Figure 3 and SI Figure S20). The positions of these fragments,

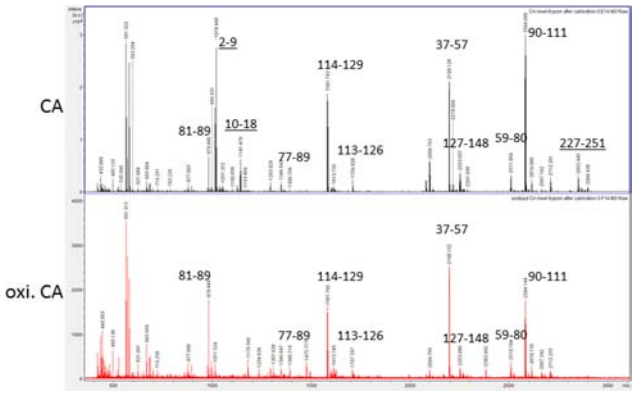


Figure 3. MALDI-TOF MS analysis after tryptic digestion of intact CA and CA oxidized by light-activated **3**. The peak patterns of the detected fragments of intact CA (upper) and oxidized CA (lower) are shown.

which include histidine, methionine, or tryptophan, are close to the ligand-binding site of CA inhibitors according to the crystal structure of CA[18] (Figure 4: binding model of **3**). These results

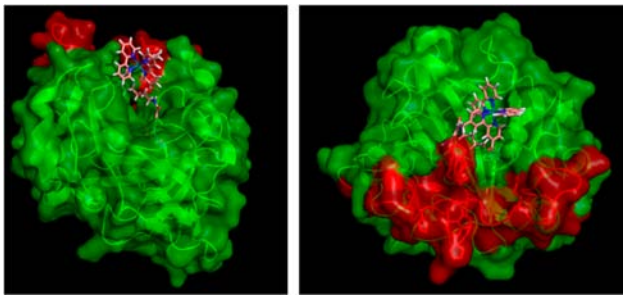
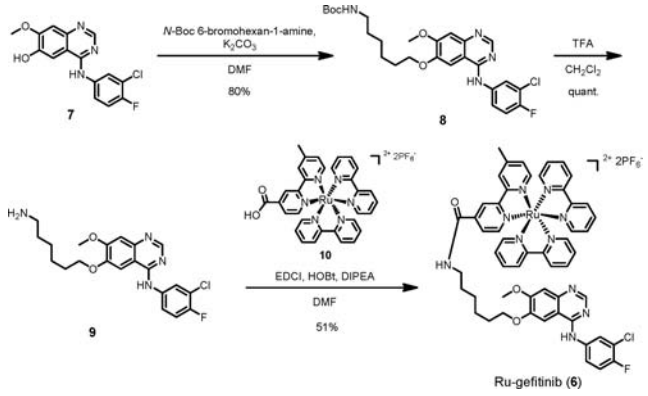


Figure 4. Binding model of CA with **3** was constructed based on the X-ray crystal structure of CA inhibitor having benzensulfonamide moiety (PDB: 4ILX) on Pymol software. The 3D structure of **3** (pink sticks) was calculated on Chem3D Pro using MM2 method, and overlapped with the inhibitor structure in reported 3D structure. Red region was the oxidized fragments 2−18 and 227−251.

suggest that **3**, with light irradiation, induced the oxidation around the binding site on CA in the absence of TRT, resulting in the light-inactivation of CA.

Regulation of Epidermal Growth Factor Receptor Knockdown and Labeling in A431 Cells. The regulation of target-selective CALI and biotin labeling in cells was examined using the epidermal growth factor receptor (EGFR) as the target protein. A431 cells, which overexpress EGFR in their membranes, were used for the experiments. Gefitinib (**5**), an EGFR tyrosine kinase inhibitor, was selected as the ligand of EGFR,[19] and Ru-gefitinib (**6**) was designed and synthesized as the EGFR-targeting Ru catalyst (Scheme 1; see Experimental

Scheme 1. Synthesis of Ru-gefitinib (6)



Procedures). Ru-gefitinib (**6**) inhibited the phosphorylation of EGFR (IC_{50} = 2.65 μM) in the A431-cell-based assay (SI Figure S21). Although the biological activity was degreased by the introduction of photocatalyst part (IC_{50} of **5** = 1.85 nM), due to the bulkiness of $Ru(bpy)_3$, **6** still has an EGFR binding activity in A431 cells. After treatment with **6**, the A431 cells were irradiated with visible light for 30 min. Figure 5B shows an

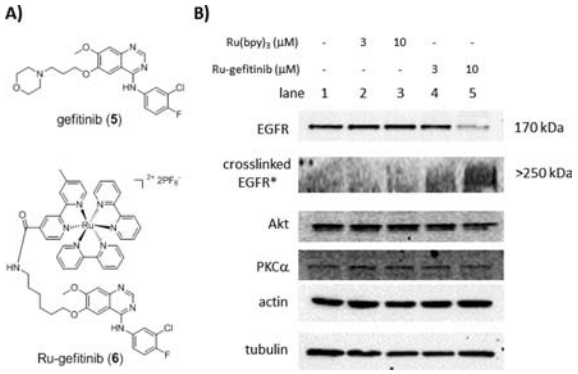


Figure 5. EGFR knockdown using Ru-gefitinib (**6**) in A431 cells. (A) Chemical structures of gefitinib (**5**) and Ru-gefitinib (**6**). (B) Immunoblotting analysis of EGFR, Akt, PKCα, actin, and tubulin under various conditions. A431 cells were irradiated with visible light for 30 min after treatment with the $Ru(bpy)_3$ complex (lanes 2 and 3) or **6** (lanes 4 and 5). *Cross-linked EGFR was detected under the longer exposure condition using the same anti-EGFR antibody.

immunoblotting analysis of EGFR, Akt, PKCα, actin, and tubulin under various conditions. The detected levels of EGFR protein decreased in the presence of **6** in a concentration-dependent manner, without affecting the levels of the other proteins (Figure 5B lanes 4 and 5; also see SI Figure S22). This reduction was not observed in the presence of the $Ru(bpy)_3$ complex (lanes 2 and 3). Instead of a reduction in the band corresponding to the EGFR monomer, a ladder of bands with higher molecular weights was observed with the anti-EGFR antibody, suggesting that light-activated **6** induced the oxidative oligomerization of EGFR or its cross-linking with other proteins. The tyrosyl radical induced by the light-activated

DOI: 10.1021/bc500518t
Bioconjugate Chem. 2015, 26, 250−256

$Ru(bpy)_3$ complex is reported to cause tyrosine−tyrosine cross-linking among the interacting proteins.[11] The cross-linking between EGFR and its interacting partner proteins might be induced simultaneously with EGFR oxidation.

The knockdown of EGFR protein by **6** was dependent on the binding between the gefitinib motif and the EGFR tyrosine kinase domain, and was markedly regulated by the addition of TRT; EGFR knockdown was inhibited in the presence of TRT (**1**) (SI Figures S23 and S24) and EGFR labeling was induced in the presence of biotin-TRT (**4**) (SI Figures S25 and S26). The period of irradiation with visible light was also an important factor in both EGFR protein knockdown and labeling. The monomeric EGFR band decreased as the period of irradiation increased in the absence of **4** (Figure 6; lanes 2, 5, and 8). EGFR labeling was affected by both the irradiation time (lanes 4, 7, and 10) and the concentration of **4** (lanes 5−7 and 8−10). Indeed, EGFR knockdown was strongly inhibited and remarkable labeling was observed with 500 μM of **4** (lanes 7 and 10). Off-target proteins (150 and 250 kDa) were also detected under the labeling conditions (not identified).

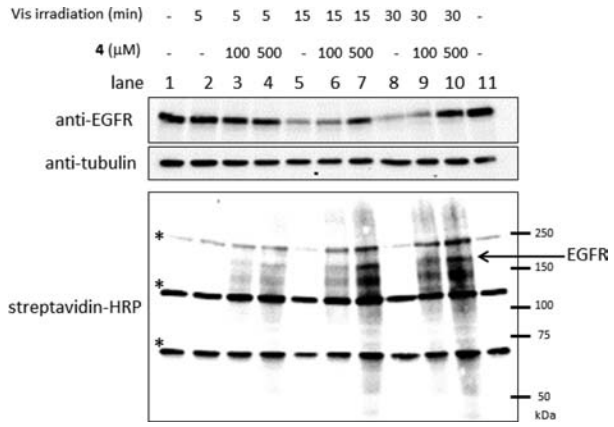


Figure 6. Effects of the period of visible light irradiation and the concentration of biotin-TRT (**4**) on the regulation of EGFR protein knockdown and labeling. The reaction was performed in the presence of 10 μM Ru-gefitinib (**6**). *Endogenous biotinylated proteins.

CONCLUSIONS

We have demonstrated the protein knockdown mechanism catalyzed by a ligand-directed $Ru(bpy)_3$ photocatalyst under visible light irradiation. Histidine, methionine, and tryptophan residues were oxidized by the $^1O_2$ generated by the light-activated $Ru(bpy)_3$ complexes. CA-selective protein knockdown was achieved using a CA-ligand-conjugated $Ru(bpy)_3$ complex. Specific regions of CA close to the ligand-binding site are thought to be oxidized, inactivating CA. We also synthesized a gefitinib-conjugated $Ru(bpy)_3$ complex as an LSC and achieved EGFR-selective knockdown in A431 cells. TRT regulated both the target protein knockdown and labeling. CA and EGFR knockdown were inhibited and labeling proceeded in the presence of TRTs. This is the first example of the regulation of both protein knockdown and labeling by a single photocatalyst. Although target-selective CALI in cells was achieved using light-activated $Ru(bpy)_3$ complexes,[6] our LSC system allows us to conduct both target-protein-selective knockdown and labeling readily with or without the use of TRT in cells. In this context, the LSC system is applicable not only to the identification of target proteins, but also to the knockdown of compound-binding proteins. Target protein identification and knockdown using LSC are now being studied in our laboratory.

EXPERIMENTAL PROCEDURES

General. NMR spectra were recorded on a VARIAN UNITY-INOVA 400 (400 MHz) spectrometer for ^1H NMR spectra and a JEOL JNM-AL 300 (300 MHz) spectrometer for ^{13}C NMR spectra. ^1H NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 7.26), acetonitrile-d_4 (δ 1.93). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), br (broad), or m (multiplet). ^{13}C NMR chemical shifts are reported as δ in units of parts per million (ppm) relative to $CDCl_3$ (δ 77.0), acetonitrile-d_4 (δ 118.2). The chemical shifts are reported in δ units relative to internal tetramethylsilane. IR spectra were recorded on a Shimadzu FTIR-8200A spectrometer. High-resolution mass spectra (ESI) were recorded on a Bruker Daltonics micro TOF-15 focus. MALDI-TOF mass analyses were performed by a Shimadzu AXIMA Performance, Shimazu AXIMA-CFR Plus or Bruker, UltrafleXtreme. LC-ESI analyses were performed by Bruker ESI-TOF-MS (micOTOF II) and VIOLAMO200 C18 HPLC column (2.1 mm I.D. × 250 mm). Analytical thin layer chromatography (TLC) or preparative TLC (PTLC) was performed on a glass plate of silica gel 60 GF254 (Merck). Column chromatography was conducted on silica gel (Merck Kieselgel 70−230 mesh). Reverse phase recycle gel permeation chromatography was performed by a Japan Analytical Industry JAIGEL-GS310. Reverse phase column chromatography was performed with GL Science InterSep C18. Most commercially supplied chemicals were used without further purification. All chemicals and purified proteins for biological experiments were obtained from commercial sources and used without further purification.

Synthesis of Compounds. *N*-(4-Dimethylaminophenyl)-acetamide (TRT, **1**), bis(2,2′-bipyridine)-(4′-methyl-*N*-(6-(4-sulfamoylbenzamido)hexyl)-[2,2′-bipyridine]-4-carboxamide)-ruthenium(II) bis(hexafluorophosphate) (Ru-SO_2NH_2, **3**), N^5-(Biotin-polyethylene glycol)-N^1-(4-(dimethylamino)phenyl) glutaramide (biotin-TRT, **4**), and **10** were prepared according to the previously reported procedures.[9]

6-((6-Aminohexyl)oxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxyquinazolin-4-amine (9). Compound **7** was prepared according to a previously reported method by Cai et al.[20] To a solution of **7** (200 mg, 0.626 mmol) in DMF (5 mL), *N*-Boc 6-bromohexan-1-amine (351 mg, 1.26 mmol), and K_2CO_3 (435 mg, 3.15 mmol) were added, and stirred at r.t. for 12 h. The reaction mixture was quenched with H_2O, extracted with AcOEt, and washed with brine. The organic layer was evaporated and the resulting solid was filtered washing with hexane afforded crude **8** (261 mg). The product (150 mg) was dissolved in CH_2Cl_2 (3 mL), TFA added (0.43 mL) at 0 °C, and stirred at r.t. for 3 h. A solution of sat. $NaHCO_3$ aq. was added to become basic. Compound **9** was obtained after extraction with AcOEt and removal of solvent (135 mg, 2 steps 81%). ^1H NMR (400 MHz; $CDCl_3$) δ 8.65 (s, 1H), 7.88 (dd, J = 6.4, 2.8 Hz, 1H), 7.55 (m, 1H), 7.48 (br, 1H), 7.25 (s, 1H), 7.16 (t, J = 8.6 Hz, 1H), 7.11 (s, 1H), 4.11 (t, J = 6.8 Hz, 2H), 4.00 (s, 3H), 2.72 (t, J = 6.6 Hz, 2H), 1.97−1.90 (m, 2H), 1.57−1.41 (m, 8H).

Bis(2,2′-bipyridine)-(*N*-(6-((1-((3-chloro-4-fluorophenyl)amino)-6-methoxyisoquinolin-7-yl)oxy)-hexyl)-4′-methyl-[2,2′-bipyridine]-4-carboxamide)

DOI: 10.1021/bc500518t
Bioconjugate Chem. 2015, 26, 250−256

Ruthenium(II) bis(hexafluorophosphate) (Ru-gefitinib, 6). To a solution of **9** (20.0 mg, 0.048 mmol) and **10** (41.4 mg, 0.045 mmol) in DMF (2.0 mL) were added EDCI (26.0 mg, 0.14 mmol), HOBt (13.4 mg, 0.99 mmol), and DIPEA (31.0 μL, 0.18 mmol). The reaction mixture was stirred at r.t. for 12 h under shading condition. Compound **6** was roughly purified by with reverse phase chromatography (GL Science InterSep C18, 0−60% acetonitrile/H_2O) detecting a part of fractions by MALDI-TOF MS. Purified **6** was obtained with Recyle Gel Permeation Chromatography (JAIGEL-GS310, 60 mM KPF_6 in MeOH) and desalting by GL Science InterSep C18 (30.5 mg, 51%). ^{1}H NMR (400 MHz; acetonitrile-d_4) δ 8.73 (s, 1H), 8.53−8.46 (m, 5H), 8.45 (s, 1H), 8.10−8.03 (m, 5H), 7.85 (d, J = 5.9 Hz, 1H), 7.76−7.62 (m, 2H), 7.57 (d, J = 5.9 Hz, 1H), 7.47 (m, 1H), 7.46−7.37 (m, 5H), 7.30−7.22 (m, 2H), 7.16 (s, 1H), 4.18 (t, J = 6.6 Hz, 2H), 3.93 (s, 3H), 3.47−3.42 (m, 2H), 2.54 (s, 3H), 1.93−1.85 (m, 2H), 1.72−1.47 (m, 8H); ^{13}C NMR (75 MHz; acetonitrile-d_4) δ 164.1, 158.8, 157.9, 157.8, 157.4, 156.8, 156.3, 153.2, 153.0, 152.6, 152.5, 151.7, 151.6, 150.1, 146.5, 143.6, 138.7, 137.0, 129.6, 128.5, 128.4, 126.3, 125.3, 124.9, 123.2, 123.1, 122.3, 117.4, 117.1, 109.7, 107.3, 102.7, 102.2, 70.1, 56.7, 40.7, 29.7, 29.5, 21.3, 14.1; IR (KBr) 3636, 3418, 3088, 2934, 2855, 2496, 1621, 1578, 1500, 1466, 1427, 1216, 1068 cm^{-1}; HRMS (ESI, positive): m/z calcd. for $C_{53}H_{48}ClFN_{10}O_3Ru$ $[M]^{2+}$: 514.1313; found: 514.1315; m.p.: 183−185 °C.

General Method for Peptide Oxidation/Labeling. Angiotensin II, bradykin, and melittin was purchased from Sigma-Aldrich, and angiotensin II variants were customized by GeneScript Inc. To a solution of peptide (final concentration 100 μM) in 10 mM MES buffer (pH 6.0) was added $Ru(bpy)_3Cl_2$ (from 100 mM solution in DMSO, final concentration 1 mM). The solution was briefly vortexed and irradiated with the light (B-100A, UV−vis, 150 mW/cm^2) on ice 5 cm from light source for 15−60 min. For the labeling, TRT (**1**) added (from 100 mM solution in DMSO, final concentration 500 μM) before light irradiation. The reaction was immediately quenched with DTT (from 1 M solution in water, final concentration 10 mM) and mixed with internal standard, angiotensin I (1.0 equiv for angiotensin II, its variants, and melittin or 0.5 equiv for bradykinin). The 100× diluted reaction mixture with 0.1% TFA 0.5 μL was mixed with 0.5 μL of CHCA solution (0.5 mg/mL solution in acetonitrile: 0.1% TFA = 1:1) on MALDI-TOF plate and dried at room temperature. The modified protein peaks were detected with MALDI-TOF analysis (Shimadzu AXIMA Performance or Shimadzu AXIMA-CFR) and peaks was analyzed as shown in SI Figures S1−S3, S5−S12, S15, and S16.

General Method for LC-ESI Analysis. To a solution of peptide (final concentration 100 μM) in 10 mM MES buffer (pH 6.0) was added $Ru(bpy)_3Cl_2$ (from 100 mM solution in DMSO, final concentration 1 mM). The solution was briefly vortexed and irradiated with light (B-100A, UV−vis, 150 mW/cm^2) on ice 5 cm from the light source for 15−60 min. For the labeling, TRT (**1**) added (from 100 mM solution in DMSO, final concentration 500 μM) before light irradiation. The reaction was immediately quenched with ascorbic acid (from 1 M solution in water, final concentration 10 mM) and briefly vortexed. The reaction mixture was analyzed by LC-ESI performed with Bruker ESI-TOF-MS (micOTOF II) and VIOLAMO200 C18 HPLC column (2.1 mm I.D. × 250 mm). The micropump gradient method was used, as follows. Mobile phase A: 0.1% FA; mobile phase B: 100% MeCN. 0−2.5 min: 5% B, 2.5−45 min: 5−60% B, 45−50 min: 60−100% B, 50−55 min: 100% B, 55.1−65 min: 5% B.

Detection of Singlet Oxygen. To a solution of SOSG (from 1 mM solution in DMSO, final concentration 100 μM) in 10 mM MES buffer (pH 6.0) was added $Ru(bpy)_3Cl_2$ (from 100 mM solution in DMSO, final concentration 1 mM). The solution was briefly vortexed and irradiated with visible light on ice 5 cm from light source (GENTOS MegaFire MF-1000D, 1000 lm LED light) for 1 or 5 min (5 min for SI Figures S13, S14). The reaction mixtures were transferred to a 96 well black plate. The fluorescence intensity (Ex: 485 nm, Em: 535 nm) was measured with plate reader (TECAN, infinite F200). See SI Figures S13, S14, and S18.

General Method for the Protein Knockdown and Labeling Experiments in a Mixture of BSA and CA. To a mixture of BSA (Sigma-Aldrich) and bovine CA II (Sigma-Aldrich) (final concentration 5 μM: 5 μM) in 10 mM MES buffer pH 6.0 was added $Ru(bpy)_3Cl_2$ or Ru-SO_2NH_2 (**3**) (from 1 mM solution in DMSO, final concentration 10 μM) in the presence or absence of biotin-TRT (**4**) (from 100 mM solution in DMSO, final concentration 1 mM), briefly vortexed and incubated at r.t. for 5 min.. After adding ammonium persulfate (APS) (from 100 mM solution in water, final concentration 1 mM), the reaction mixture was light-irradiated (B-100A, UV−vis, 150 mW/cm^2) on ice 5 cm from light source for 30 min. For the competition experiment, **2** (from 1 M solution in DMSO, final concentration 1 mM) was added and preincubated for 10 min before addition of **3** against the mixture of BSA and CA. The reaction was quenched by removal of excess **4** by Bio-Spin 6 column (Bio rad). The amount of proteins was analyzed with CBB stain after separation with SDS-PAGE (12.5% acrylamide gel). For the detection of biotin-labeled proteins, separated proteins with SDS-PAGE were transferred to a PVDF membrane (GE Healthcare). The membrane was blocked with Immuno Block (DS Pharma) and treated with horseradish peroxidase (HRP)-conjugated streptavidin (streptavidin-HRP, Sigma-Aldrich), the blot was treated with ECL kit (GE Healthcare). The chemiluminescence images were obtained with a Molecular Imager ChemiDoc XRS System (Bio rad).

CA Knockdown. CA (10 μM) in 10 mM MES buffer (pH 6.0) was incubated with **3** (3 or 10 μM) at r.t. for 30 min. After the incubation, to each reaction mixture (10 μL/well in 96 well plate) was added 4-nitrophenyl acetate (1.1 mM in Tris buffer 50 mM, pH 8.0, 90 μL/well in 96 well plate) and the kinetic change of OD360 was measured with plate reader (TECAN, infinite F200). See SI Figure S19.

Triptic Digestion of CA Oxidized by Light Activated $Ru(bpy)_3$. CA was oxidized under the condition of Figure 2 lane 3 (see the general method for the protein knockdown and labeling experiments in a mixture of BSA and CA in the Experimental section). The separated CA protein in SDS-PAGE was digested in gel using Trypsin digestion kit for MS analysis (MIXELL, APRO SCIENCE). The peptide fragment mixture was purified on C18 Zip Tip to remove salt. The peptides were eluted with 50% acetonitrile/0.1% TFA aq. The eluted peptides were mixed with 1 μL of CHCA solution (0.5 mg/mL solution in acetonitrile:0.1% TFA = 1:1) on MALDI-TOF plate and dried at room temperature. The modified protein peaks were detected with MALDI-TOF analysis (Bruker, UltrafleXtreme).

EGFR-Selective Knockdown/Labeling in A431 Cells. A431 cells were cultured under 5% CO_2 at 37 °C in RPMI-

DOI: 10.1021/bc500518t
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1640 media (Wako pure Chemical) containing 10% fetal bovine serum (FBS, Hyclone, Logan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The cells were seeded at a density of 2.0×10^5 cells media in a 35 mm plastic dishes (Greiner) with 2 mL media/dish, and incubated under 5% CO_2 at 37 °C for 48 h. The cells were washed with PBS and the media were replaced by MES-buffered saline (MBS, 10 mM MES, 150 mM NaCl, pH 6.0), added Ru-gefitinib (**6**) or $Ru(bpy)_3Cl_2$ (from 10 mM solution in DMSO, final concentration 10 μM) with or without bitoin-TRT (**4**) (from 100 mM solution in DMSO, final concentration 500 μM) and incubated under 5% CO_2 at 37 °C for 1 h. Each dish was irradiated with visible light on ice 10 cm from light source (GENTOS MegaFire MF-1000D, 1000 lm LED light) for 5–30 min. After irradiation, cells were washed with PBS and lysed with 100 μL lysis buffer/dish. Excess amount of **4** and Ru complexes was removed by Bio-Spin 6 (Bio rad). The resulting samples were added to 5× SDS-PAGE sample buffer and heated at 95 °C for 5 min. The proteins were separated by SDS-PAGE with 8% acrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare), blocked with Immuno Block (DS Pharma), and immunoblotted with anti-EGFR (Santa Cruz), anti-Akt (Cell Signaling Technology), anti-PKCα (Cell Signaling Technology), anti-actin (Sigma-Aldrich), or anti-tubulin (Santa Cruz). After further incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz), the blot was treated with ECL kit (GE Healthcare) and protein expression was visualized with a Molecular Imager ChemiDoc XRS System (Bio rad). For the detection of biotin-labeled proteins, streptavidin-HRP (Sigma-Aldrich) was used instead of antibodies.

EGFR Tyrosine Kinase Inhibitory Assay in A431 Cells.[21] A431 cells were seeded at a density of 2.0×10^5 cells media in a 35 mm plastic dishes (Greiner) with 2 mL media/dish, and incubated under 5% CO_2 at 37 °C for 36 h. The media were replaced by FBS-free RPMI-1640 and incubated 5% CO_2 at 37 °C for 12 h. Each concentration of gefitinib (**5**) or Ru-gefitinib (**6**) was added and incubated under 5% CO_2 at 37 °C for 2 h. After incubation of compounds, cells were incubated with hEGF (Sigma-Aldrich, final concentration 10 ng/mL) for 5 min, and lysed with 100 μL lysis buffer/dish. Phosphorylated EGFR was detected with antiphospho-EGFR (Santa Cruz), and other proteins were detected by above-mentioned method. See SI Figure S21.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products (26102721 to H.N.)" and "Young Scientist (B) (25810104 to S.S.)" from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

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DOI: 10.1021/bc500518t
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