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Photoactivation of anticancer Ru complexes in deep tissue: How deep can we go?

Wen Sun,[a] Raweewan Thiramanas,[a] Leonardo D. Slep,[c] Xiaolong Zeng,[a] Volker Mailänder*[a][b] and Si Wu*[a]

Abstract: Activation of anticancer therapeutics such as ruthenium (Ru) complexes is currently a topic of intense investigation. The success of phototherapy relies on photoactivation of therapeutics after the light passes through skin and tissue. In this paper, we study the photoactivation of anticancer Ru complexes with 671-nm red light through tissue of different thicknesses. We synthesized four photoactivatable Ru complexes with different absorption wavelengths. Two of them (Ru3 and Ru4) were responsive to wavelengths in the "therapeutic window" (650-900 nm) and could be activated using 671-nm red light while the other two (Ru1 and Ru2) could not be activated using red light. We demonstrated that 671-nm light can activate Ru3 and Ru4 after passing through tissue up to 16-mm-thick. Furthermore, after passing through an 8-mm-thick tissue, 671-nm light activated Ru4 and caused inhibition of cancer cells. These results suggest that photoactivatable Ru complexes are promising for applications in deep-tissue phototherapy.

Introduction

Phototherapy based on phototherapeutic agents and light irradiation is a promising strategy for cancer treatment.[1] Phototherapeutic agents are usually non-toxic or less toxic in the dark, until light converts them to toxic species that kill cancer cells (Figure 1a). Phototherapy causes minimal side effects for normal tissues because light provides high spatial resolution and allows activation of the phototherapeutic agents at target sites only.[1-4] Most photoresponsive materials,[5-6] such as photoactivated platinum,[7-8] coumarin-caged [9-10] and pyrene-caged[11] prodrugs are sensitive to UV or short-wavelength visible light. However, UV or short-wavelength visible light is problematic for biomedical applications, because these wavelengths cannot penetrate deeply into tissue.[12] That is beside the fact that, UV light can damage biological systems. Compared with UV or short-wavelength visible light, red or near-infrared (NIR) light is thus better suited to biomedical applications, because red or NIR light has a deeper tissue penetration depth (Figure 1b).[12-13]

Simultaneous two-photon absorption is one way to activate photoresponsive materials using NIR light.[14-17] However, two-photon absorption is inefficient and only occurs at the focus of high-intensity pulsed lasers.[18-19] Because a laser beam will defocus while passing through tissue, a two-photon absorption strategy is impractical for deep-tissue applications. Another method for activating phototherapeutic agents using NIR light is based on photon upconversion. NIR light can be converted by upconverting nanoparticles or some organic dyes to UV or visible light, which then activates phototherapeutic agents.[12,20-23] This process is referred to as upconversion-assisted photochemistry.[12] Compared to simultaneous two-photon excitation, one advantage of upconversion-assisted photochemistry is that it does not require high-intensity pulsed lasers. However, upconversion is still a non-linear optical process and requires high-intensity laser excitation (at least several hundred mW/cm^2), which may damage biological systems.[12,24] Furthermore, although NIR light can penetrate into tissue deeply, passing through tissue still attenuates its intensity. Thus, the NIR light intensity may be too low to excite upconversion after NIR light passes through relatively thick tissue.

Activation of phototherapeutic agents directly using red or NIR light via a one-photon process is more efficient than nonlinear optical processes such as two-photon absorption and photon upconversion. Some phototherapeutics that can be directly activated by red or NIR light via a one-photon process already exist.[25-29] Previous studies have demonstrated that Ru complexes are photoresponsive molecules and have been applied for biological applications.[30-33] In particular, our group and others show that some ruthenium (Ru) complexes can be directly activated by low-intensity (30-720 mW/cm^{-2}) red or NIR light.[34-37] Ru complexes, analogues of platinum anticancer drugs, are importantly proposed to also be promising anticancer agents.[26, 38-39] One advantage of photoactivated Ru complexes is that they are usually less toxic to non-irradiated tissues, only becoming more toxic in tumor cells through photoactivation.[14, 26, 39-47] Photoactivated Ru-containing materials have already shown anticancer effects in a tumor-bearing mouse model.[34,37,48] Further, photoactivated Ru complexes are promising for deep-tissue phototherapy due to their high photo-responsiveness

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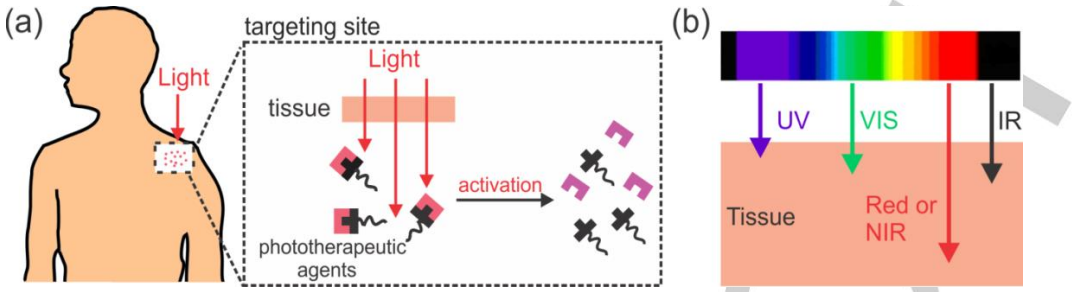

Figure 1. Schematic illustrations: (a) Light passes through tissue and activates phototherapeutic agents. (b) Tissue penetration depth of light with different wavelengths. Red or NIR light has a deeper tissue penetration depth than UV and visible light. When the light wavelength further shifts to the IR region, the penetration depth decreases again because water absorbs IR light.

(e.g. several mW/cm^{-2}). However, red or NIR light will eventually be completely attenuated while passing through thick tissue, which means photoactivation of therapeutics via one-photon processes has a certain depth limit. The understanding of this photoactivation of therapeutics in deep tissue will thus help provide guidelines for phototherapy.

Here, we systematically investigated the photoactivation of anticancer Ru complexes after light passes though tissue with different thicknesses. We demonstrated that light is able to pass through 16-mm-thick tissue and activate two Ru complexes. Our results thus indicate phototherapy using Ru complexes is a promising for biological systems with a tissue thickness on the order of 16 mm.

Results and Discussion

To study photoactivation, we synthesized four photocleavable Ru complexes (Figure 2a): $[Ru(bpy)_2(CH_3CN)_2]^{2+}$ (Ru1), $[Ru(tpy)(bpy)(CH_3CN)]^{2+}$ (Ru2), $[Ru(tpy)(biq)(CH_3CN)]^{2+}$ (Ru3) and $[Ru(biq)_2(CH_3CN)_2]^{2+}$ (Ru4) (bpy = 2,2'-bipyridine; tpy = 2,2':6',2''-terpyridine; 2,2'-biquinoline). The Ru-acetonitrile coordination bonds in all the complexes are photocleavable (Figure S1). However, the wavelengths for photocleavage of these Ru complexes are different because their metal-to-ligand charge transfer (MLCT) bands are located at different wavelengths (Figure 2b). The absorption maxima of Ru1 (black line) and Ru2 (blue line) were 425 nm and 455 nm, respectively, while the absorption tails of Ru1 and Ru2 terminated at ~550 nm and ~605 nm, respectively. Therefore, red to NIR light in the "therapeutic window" (e.g., 650-900 nm) was unable to trigger the photocleavage of Ru1 and Ru2 (Figure S2 and S3). The absorption maxima of Ru3 (green line) and Ru4 (red line) were located at 515 nm and 535 nm with absorption tails up to ~750 nm and ~780 nm, respectively. Further, the MLCT bands of Ru3 and Ru4 did not change when they were kept in the dark for an hour, indicating both complexes were thermally stable within the experimental time period. Red light irradiation (671 nm, 110 mW/cm^2) successfully induced the photocleavage of Ru3 and Ru4 (Figure S4 and S5). These results showed that Ru3 and Ru4 are good model compounds to investigate photoactivation in deep tissue.

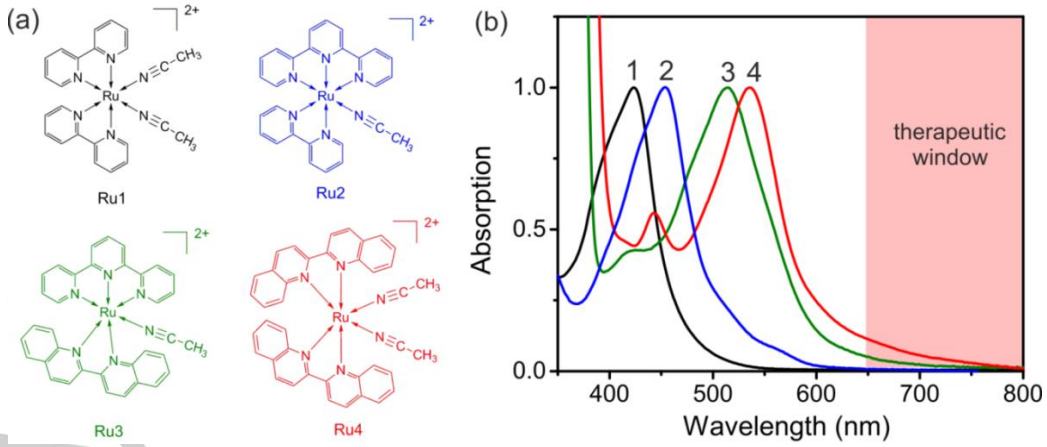

Figure 2. (a) Chemical structures of four photoactivatable Ru complexes Ru1-Ru4. (b) UV-Vis absorption spectra of Ru1-Ru4. Red region represents the "therapeutic window".

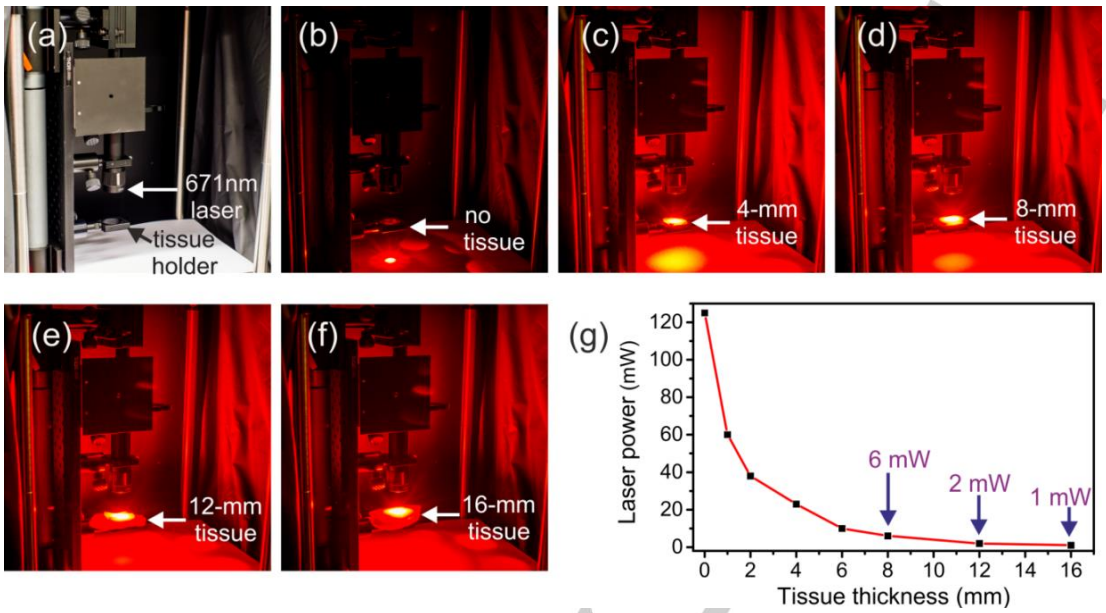

Figure 3. (a) Photograph of the laser setup. (b)-(f) Photographs showing the laser (671 nm, 125 mW) passing through pork tissue with different thicknesses: (b) no tissue, (c) 4-mm tissue, (d) 8-mm tissue, (e) 12-mm tissue and (f) 16-mm tissues. (g) Laser power after the laser (671 nm, 125 mW) passed through pork tissue with different thicknesses.

We first characterized the ability of 671-nm light to penetrate tissue, which was used for photoactivation of Ru3 and Ru4. We measured the laser power after the laser passed through tissue with different thicknesses, using the setup in Figure 3a. A DPSS (diode pumped solid state) laser at 671 nm was used as the light source. A tissue holder with a circular hole in the center was placed vertically below the laser. The laser was a parallel beam with an intensity 110 mW/cm^2 (Figure 3). This light intensity was chosen for our experiments because the maximum permissible exposure for skin at 671 nm is 0.2 W/cm^2 according to the American National Standard for Safe Use of Lasers.[49] Thus, we want to use such an intensity to enable activation of Ru complexes while preventing photodamage to tissues. After passing through a 4-mm-thick pork tissue, the luminance of the laser spot became weak due to strong light scattering, reflection, and a little absorption (Figure 3c). The luminance of the laser spot gradually decreased when the tissue thickness increased (8 mm in Figure 3d and 12 mm in Figure 3e). When the tissue was as thick as 16 mm, the luminance of laser spot was comparable to that of the surrounding (reflected and scattered light) and the laser spot was nearly invisible to "the naked eye" (Figure 3f). These results clearly demonstrated thickness strongly influenced laser power after passage through the tissue (Figure 3b-f). To quantify this intuitive observation, we used a power meter to measure the laser power after passing through tissues (Figure 3g). The laser power was 60, 38, 23, 10, 6, 2, and 1 mW after passage through 1, 2, 4, 6, 8, 12, and 16 mm thick pork tissue. Thus, while 671 nm light can penetrate deeply into tissue, only low laser powers can be obtained deep inside the tissue. Therefore, highly photosensitive materials, which are responsive to low-intensity red light, are best suited for deep-tissue phototherapy.

UV-Vis absorption spectroscopy was employed to follow the photocleavage of Ru3 (Figure 4a). Irradiating (671 nm, 125 mW) an aqueous solution of Ru3 for 2 min resulted in a decrease of the MLCT band at 515 nm and the appearance of a new peak at 550 nm (Figure 4b). Prolonged irradiation resulted in negligible spectral changes, suggesting the photoreaction was fast and completed within 2 min. The quantum yield of the photoreaction (Φ_{Ru3}) induced by 671 nm irradiation was calculated to be 0.47. Then, we systematically investigated the photoreaction of Ru3 after the laser passed through pork tissue with different thicknesses (1-16 mm). Light irradiation in the presence of pork tissue (1-16 mm thick) still resulted in the change of the MLCT band, showing that Ru3 can be photoactivated in deep tissue (Figure 4c-f). However, different irradiation times were required to complete the photoreaction. The reaction was completed in 9, 24, and 58 min when the laser passed through 1-mm, 4-mm, and 8-mm-thick tissue. In fact, the laser even induced the photoreaction after passing through 16-mm-thick tissue (Figure 4f). Evolution of the photoreaction represented by relative absorption changes A_t/A_0 (550 nm) shows that 1h-irradiation through 16-mm tissue resulted in ~80% relative absorption changes (Figure 4g).

We conducted a similar study of the photocleavage of Ru4 (Figure 5a). Irradiating (671 nm, 125 mW) an aqueous solution of Ru4 for 6 min resulted in a decrease of the MLCT band at 535 nm and the appearance of a new peak at 585 nm (Figure 5b). The quantum yields for the first ($\Phi_{Ru4\text{-}1}$) and second ligand ($\Phi_{Ru4\text{-}2}$) exchange of Ru4 induced by 671 nm irradiation were measured to be 0.082 and 0.017, respectively. We also

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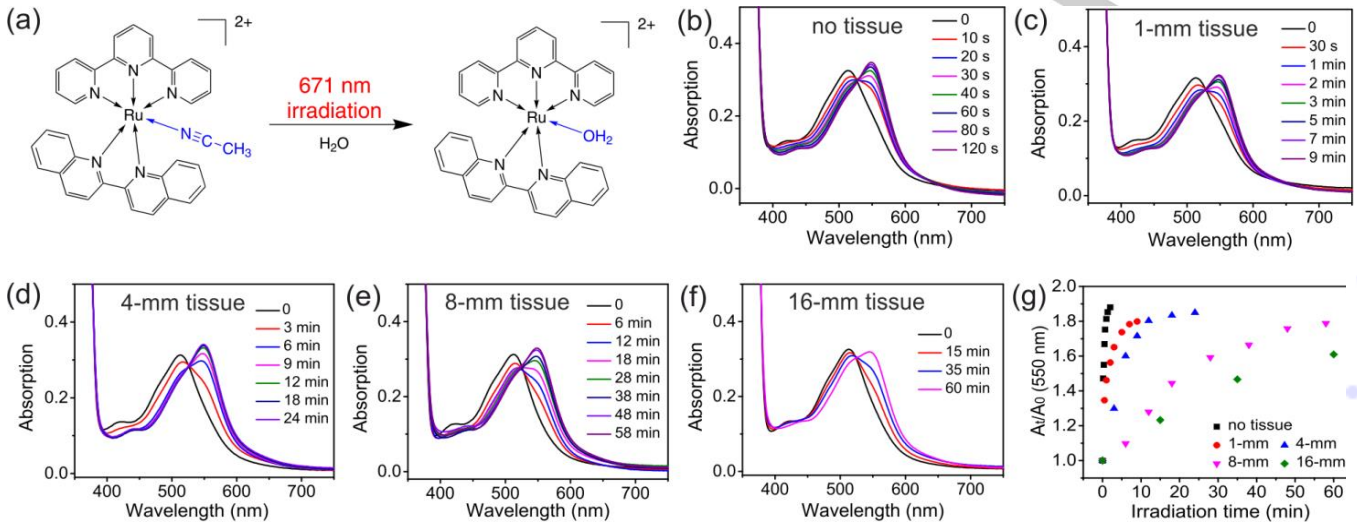

Figure 4. (a) Photocleavage of Ru3. (b)-(f) UV-Vis absorption spectra of Ru3 (6.6×10^{-5} M, H_2O) before and after irradiation (671 nm, 125 mW), (b) in the absence of tissue and in the presence of (c) 1-mm, (d) 4-mm, (e) 8-mm, and (f) 16-mm-thick pork tissues. (g) Relative absorption changes at 550 nm after irradiation in the presence of pork tissues with different thickness.

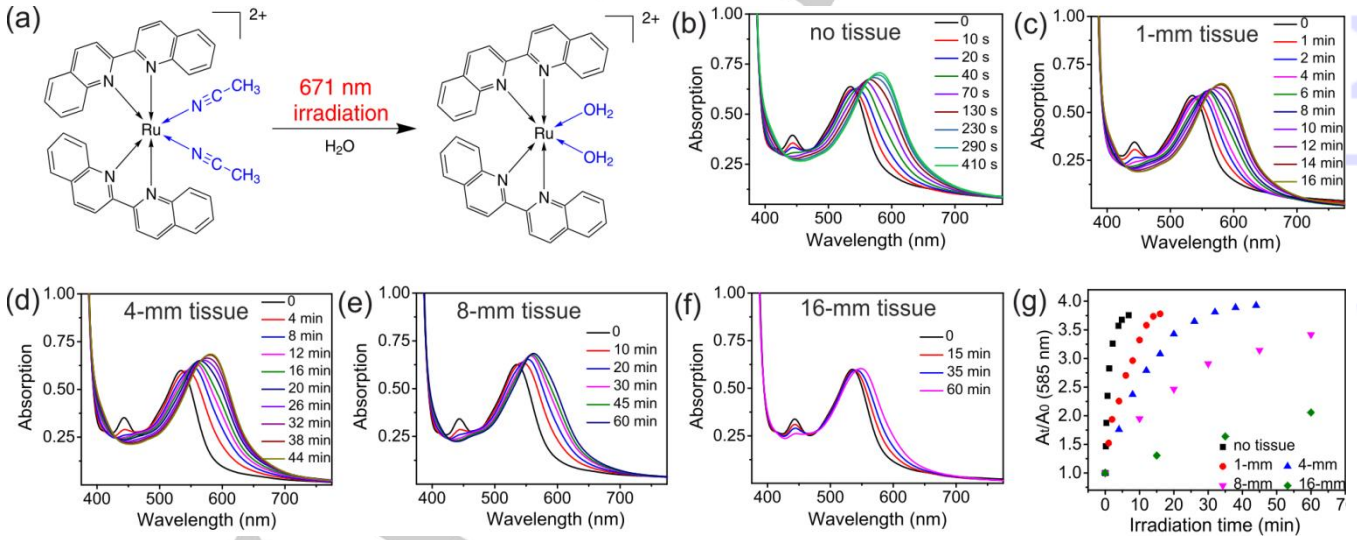

Figure 5. (a) Photocleavage of Ru4. (b)-(f) UV-Vis absorption spectra of Ru4 (9.3×10^{-5} M, H_2O) before and after irradiation (671 nm, 125 mW), (b) in the absence of tissue and in the presence of (c) 1-mm, (d) 4-mm, (e) 8-mm, and (f) 16-mm-thick pork tissues. (g) Relative absorption changes at 585 nm after irradiation in the presence of pork tissues with different thickness.

systematically investigated the photoreaction of Ru4 after the laser passed through pork tissues with different thicknesses (1-16 mm). Light irradiation in the presence of pork tissue (1-16 mm) resulted in the change of the MLCT band, showing that Ru4 can be activated in deep tissue (Figure 5c-f). The reaction was completed in 16 and 44 min when the laser passed through 1-mm and 4-mm-thick tissue, respectively. In fact, the laser could even induce the photoreaction after passing through 8-mm and 16-mm-thick tissue (Figure 5e and 5f). Evolution of the photoreaction A_t/A_0 (585 nm) shows that 1h-irradiation through 8-mm and 16-mm tissues resulted in ~80% and ~50% relative absorption changes (Figure 5g).

The studies on Ru3 and Ru4 demonstrated that red light (671 nm, 125 mW) can pass through tissue up to 16 mm and still activate them. It was reported that photoproducts from Ru complexes similar to Ru3 and Ru4 were more toxic than the Ru complexes before irradiation because the photoproducts may bind to DNA, resulting in apoptosis of cancer cells. Additionally, singlet oxygen, which is also toxic to cancer cells, can be generated during irradiation. Therefore, the combination of phototoxicity and deep-tissue photoactivation of Ru complexes is promising for deep-tissue phototherapy.

As a proof of concept, we used light passing through a piece of 8-mm-thick tissue to inhibit the growth of cancer cells in the presence of Ru4. A piece of 8-mm pork tissue was placed between the laser and HeLa cells incubated in the presence of Ru4 (Figure 6a). Without Ru4, light irradiation (λ= 671 nm, 110 mW/cm^2, 30 min) only caused a little decrease in cell viability (Figure 6b, 0 μg/mL).[50] Light irradiation (671 nm, 110 mW/cm^2, 30 min) in the presence of Ru4 caused significant cell death compared to the mere irradiation and dark condition (Figure 6b). Hence, Ru4 can be photoactivated in the presence of an 8-mm-thick tissue to inhibit the growth of cancer cells.

Conclusions

We studied the photoactivation of anticancer Ru complexes in the presence of tissues with different thicknesses. Anticancer Ru complexes (Ru1-Ru4) were irradiated using a 671-nm laser. Ru1 and Ru2 could not be activated by 671-nm light because their photoresponsive wavelengths are too short. However, Ru3 and Ru4 were activated by 671-nm light due to their long responsive wavelengths. Although only 1% of the laser power remained after passing through a 16-mm-thick tissue, light was still able to activate Ru3 and Ru4. Furthermore, phototoxicity of Ru4 was successfully induced by 671-nm light in cancer cells after passing through an 8-mm-thick tissue. Our results thus suggest that photoactivatable Ru complexes are promising for deep-tissue biomedical applications. Further red shifting the responsive wavelength of Ru complexes would be helpful for phototherapy in deeper tissue. Ru complexes responsive to 800-nm light, the best wavelength for tissue penetration, are expected to be realized in the future.

Experimental Section

Materials $RuCl_3 \cdot xH_2O$ (99.9%), 2,2':6',2''-terpyridine (>98%) and 2,2'-bipyridyl (>99%) were purchased from Alfa Aesar. 2,2'-biquinoline (98%) was purchased from Acros Organics. Lithium chloride, silver hexafluorophosphate (98%), potassium hexafluorophosphate (98%) and DOWEX 22 Cl anion exchange resin were purchased from Sigma-Aldrich All other solvents were purchased from Sigma-Aldrich or Fisher Scientific Milli-Q water with a resistivity of 18.2 MΩ·cm was used in this study. Fresh pork tissue was purchased from REWE supermarket (REWE Group, Germany).

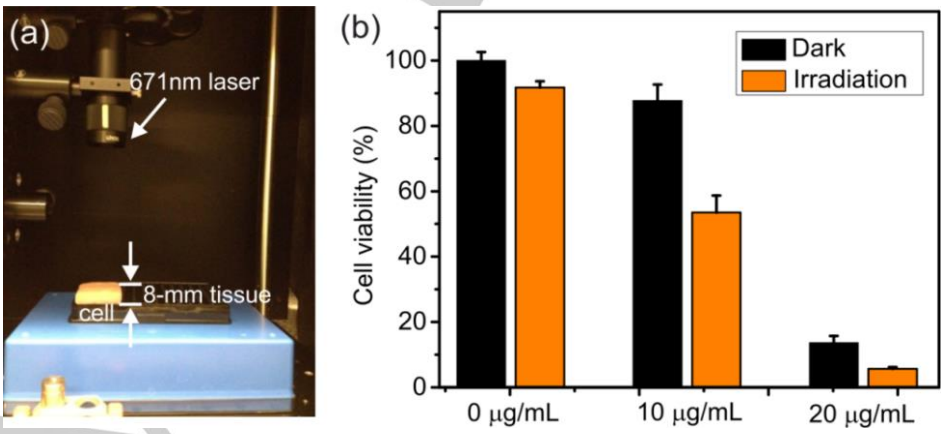

Figure 6. (a) Photograph of the setup for cell viability test. (b) Viability of HeLa cells treated with Ru4 at different concentrations in the dark and after light irradiation (λ= 671 nm, 110 mW/cm^2, 30 min). Light irradiation was performed after incubation with Ru4 for 4 h.

Methods Four Ru complexes $[Ru(bpy)_2(CH_3CN)_2](PF_6)_2$ [Ru1$(PF_6)_2$], $[Ru(tpy)(bpy)(CH_3CN)](PF_6)_2$ [Ru2$(PF_6)_2$], $[Ru(tpy)(biq)(CH_3CN)_2](PF_6)_2$, [Ru3$(PF_6)_2$] and $[Ru(biq)_2(CH_3CN)_2](PF_6)_2$ [Ru4$(PF_6)_2$] were synthesized according to the procedure described in literatures.[51-53] Notably, Ru complexes were directly precipitated from water by using saturated solution of KPF_6 followed by removing CH_3CN from the reaction solution. ^{1}H NMR and H-H COSY spectra of the Ru complexes were measured to prove the successful synthesis of the complexes (Figure S8-S16). The chloride salt of each complex was used for photoreaction experiments performed in H_2O, which were obtained using an ion exchange resin (DOWEX 22 Cl) according to a reported method.[54] Generally, 20 mg Ru complex was dissolved in 8 mL of a mixture of acetone/water (1:2) and 500 mg of anionic exchange resin (DOWEX 22 Cl) was added. The acetone was evaporated and the suspension was stirred overnight. The resulting aqueous solution was filtered to remove the resin and then dried under reduced pressure. Notably, Ru3 was found to be partly hydrolyzed after overnight stirring. Therefore, Ru3 (Cl_2) was further refluxed in CH_3CN/H_2O (50:50, v:v) for 4 h followed by removing the solvent. The chloride salt of each complex was dissolved in water in a 1 × 1 cm^2 quartz cuvette to study photolysis. UV-Vis absorption spectra were measured at room temperature using a Lambda 900 spectrometer (Perkin Elmer). A DPSS laser with a wavelength of λ=671 nm (CNI-671-200-LN-AC-3, Laser 2000 GmbH, Germany) was used as the excitation source. The laser was equipped with a thermoelectric cooling system. The output power of the DPSS laser was controlled by a tabletop laser driver (PSU-III-FDA, Changchun New Industries Optoelectronics Technology Co., Ltd., China) and measured by using an optical power meter (model 407A, Spectra-Physics Corporation). The quantum yield for the ligand exchange of Ru3 ($\Phi_{Ru3\text{-}1}$) and the quantum yields for the first ($\Phi_{Ru4\text{-}1}$) and second ligand ($\Phi_{Ru4\text{-}2}$) exchange of Ru4 were calculated according to reported method (for details see Supporting Information).[55-56]

Cell culture Hela cells obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) complete medium containing 10% fetal bovine serum (FBS, Gibco, USA), 1% L-glutamine (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) and incubated at 37°C in CO_2-incubator with 95% humidity and 5% CO_2 (C200, Labotect, Germany). To dissociate adherent cells, the cells were trypsinized with 0.25% trypsin (Gibco, USA) for 3 min as a general procedure. The cell pellet was collected by centrifugation at 130 g for 3 min, resuspended in DMEM complete medium and used for further assays. Viable cells were determined by trypan blue exclusion method and counted by using TC10™ automated cell counter (Bio-Rad, USA).

Cell viability To study cytotoxicity, HeLa cells resuspended in DMEM complete medium were seeded at a density of 6,400 cells per well in a 96-well plate for 48 h. Then, the cells were treated with Ru4 solutions at final concentrations of 10 and 20 μg/mL for 4 h prior to irradiation with a red laser (671 nm) through a sliced piece of 8 mm-thick pork tissue for 30 min. After that, the cells were further incubated at 37°C in CO_2-incubator for 24 h. Samples without light treatment were covered with aluminum foil and taken through the procedure in parallel. The sample without any treatment was used as a negative control and calculated as 100% cell viability, while 20% DMSO added sample was used as a positive control. After that, cell viability was evaluated using the CellTiter-Glo® luminescent cell viability assay (Promega, USA) according to the manufacturer's protocol. This assay is based on the amount of ATP present, which reflects the presence of metabolically active cells. Luminescence was recorded 10 min after reagent addition using plate reader (Infinite® M1000, Tecan, Germany).

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Keywords: photochemistry • Ru complex • anticancer • phototherapy • metallodrug

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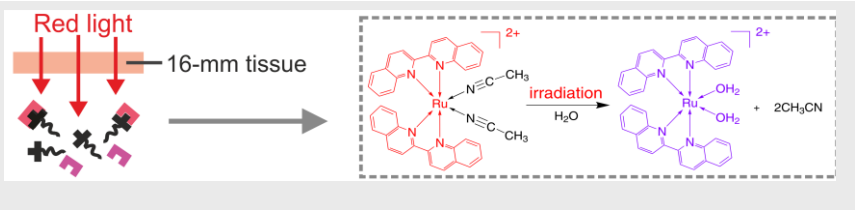

Anticancer ruthenium (Ru) complexes can be photoactivated by 671-nm light after passing through a 16-mm-thick tissue. The photoactivated Ru complexes can inhibit the growth of cancer cells. These results suggest that Ru complexes are promising anticancer agents for deep-tissue phototherapy.

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Photoactivation of anticancer Ru complexes in deep tissue: How deep can we go?