Mitochondria Targeted Protein-Ruthenium Photosensitizer for Efficient Photodynamic Applications

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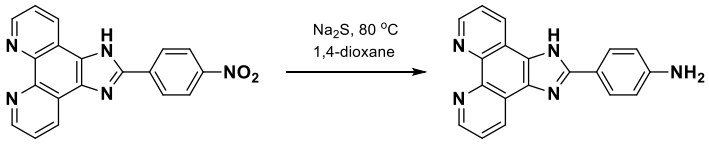
Experimental Section:

Materials and general information

cHSA and cHSA-PEO have been synthesized following the procedure reported previously by our group.[1] O-(2-Maleimidoethyl)-O'-methyl-polyethylene glycol 5000 >90% (PEO-5000-MI), N-(2-aminoethyl) maleimide trifluoroacetate salt >95% HPLC, N-hydroxy-succinimide (NHS), Formaldehyde, (3-Carboxypropyl)triphenylphosphonium bromide (TPP) 98%, 9,10-anthracenediyl-bi(methylene)dimalonic acid (ABDA, 99%) were received from Sigma-Aldrich. Na_2HPO_4, NaH_2PO_4, NaCl and $NaHCO_3$ ACS reagent grade were obtained from Goodrich Chemical Enterprise. Vivaspin ultrafiltration tubes were purchased from GE healthcare. Ultra-pure milli-Q water was applied for all experiments involving water. Emission and absorption were recorded using TECAN infinite M1000 microplate reader for small quantity sample volumes. We used JASCO V-670 UV/Vis/NIR spectrophotometer and JASCO FP-8500 fluorescence spectrometer with samples in standard quartz glass cuvettes (d=10.0 mm, Hellma) for larger quantity sample. Fluorescence quantum yields were measured in Greiner Germany 1534 well µ clear transparent micro plates. ÄKTA Explore FPLC and Superose TM 6 10/30 gel filtration columns were used for cHSA-PEO hybrid purification. Zeta-potential and DLS measurements were performed using a Malvern Zetasizer ZEN3600 (Mal-vern Ltd, Malvern, UK) at 20°C. NMR spectra were recorded on a Bruker AVANCE 400 spectrometer at ambient temperature. All spectra were referenced to the deuterated solvent as an internal standard. Chemical shifts (δ) are listed in parts per million (ppm) using the residual solvent signals (δH=2.50 ppm for DMSO-d6, δH=3.31 ppm and δC= 49.00 ppm for MeOD-d4). The molar absorption coefficient for the Ru complex was determined from dilution experiments in triplicates (three exact standard solutions of the Ru chromophore in MilliQ water). The slope of the decrease of the absorbance yielded the average ε according to the law of Lambert-Beer. For cHSA-PEO-TPP-Ru, the determination was per-formed from exact concentrations. The molar absorption coefficient was determined for Ru ($1.08 \cdot 10^4$ $L \cdot mol^{-1} \cdot cm^{-1}$) and cHSA-PEO-TPP-Ru ($0.98 \cdot 10^5$ $L \cdot mol^{-1} \cdot cm^{-1}$) at 470 nm.

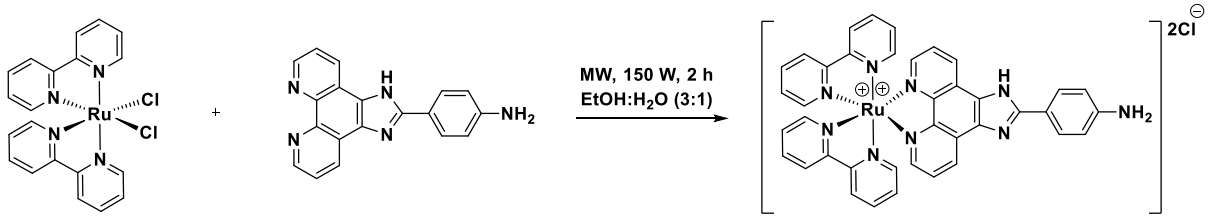
Synthesis:

Synthesis of 4-(1H-imidazo[4,5-f][1,10]phenanthroline-2-yl)-aniline



2-(4-nitrophenyl)-1H-imidazo[4,5-f][1,10]-phenanthroline (3.2 g, 9.38 mmol) was suspended in 1,4-dioxane (76 mL) and heated to 80°C. Na_2S was dissolved in water and heated to 80 °C. The warm Na_2S solution was combined with the warm yellow suspension and heated to 80 °C for 4 h. The color changed instantaneously from orange to red and the solid was dissolved completely. The dioxane was evaporated under precipitation of the product. The solid was filtered off and washed respectively with water and Et_2O. After drying the product was received as ochre colored solid in a yield of 78% (2.3 g, 7.29 mmol). ^1H NMR (400 MHz, RT, DMSO-d_6): δ = 8.90 (dd, 2 H), 8.87 (d, 2H), 8.01 (d, 2 H), 7.73 (dd, 2 H), 6.68 (d, 2H), 5.42 (s, 2 H,) ppm.

Synthesis of Ru1{[Bis(2,2'-bipyridine)-4-(1H-imidazo[4,5-f][1,10]phenanthroline-2-yl)aniline-ruthenium(II)]Cl_2}[2]

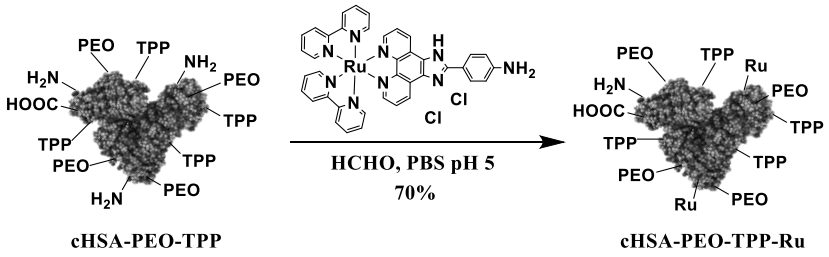


300 mg (620 µmol) $(bpy)_2RuCl_2$ and 232 mg (744 µmol) 4-(1H-imidazo[4,5- f][1,10]phenanthroline-2-yl)aniline were dissolved in EtOH (15 mL), H_2O (5 mL) and 3 drops of an aqueous KOH solution (1 plate dissolved in 15 mL H_2O) were added. The solution was reacted in the microwave for 2h at 180 W and changed the color from dark violet to red. The solvents were rotary evaporated, the residue was dissolved in EtOH, Et_2O was added and red crystals precipitated. The red crystals were filtered off. Via size exclusion chromatography (Sephadex) in MeOH the desired complex was isolated as a bright orange band. After the solvent was removed via rotary evaporation the product was isolated as red crystals (92%, 414.5 mg, 571 µmol). 1H NMR (400 MHz, RT, MeOD-d_4): δ = 9.12 (t, 2H), 8.75 (d, 2H), 8.71 (d, 2H), 8.16 (td, 2H), 8.03 (dd, 4 H), 7.97 (ddd, 4 H), 7.76 (dd, 2H), 7.67 (dd, 2H), 7.59 – 7.44 (m, 1H), 7.31 (ddd, 2H), 6.81 (d, 2H) ppm. ^{13}C NMR (100 MHz, RT, MeOD-d_4): δ = 157.55, 157.38, 152.01, 150.66, 146.14, 138.15, 138.02, 134.87, 130.68, 127.85, 127.73, 127.42, 127.21, 126.37, 124.55, 124.46 ppm. HRMS (MALDI-FT-ICR): 724.15194 $[M\text{-}H^+\text{-}2Cl^-]^+$ (calculated: 724.15057).

Synthesis of cHSA-PEO-TPP

This reaction was preceded through EDC coupling pathway. First, TPP (8 mg), NHS (3 mg) and EDC.HCl (4 mg) was dissolved and degassed in 0.5 mL of DMF solution. This mixture was stirred at room temperature under argon atmosphere for overnight. Next day, cHSA-PEO (2 mg) dissolved in 2 mL milli-Q water was added and again reacted at room temperature for overnight. Finally, the product was washed through vivaspin 20 (MWCO 30K) ultracentrifuge tube to separate unreacted reactants. Finally, it was kept at 4 °C for future use.

Synthesis of cHSA-PEO-TPP-Ru



cHSA-PEO-TPP **cHSA-PEO-TPP-Ru**

This reaction proceeded based on a slightly modified literature reported procedure.[3] To a 1.5 mL micro-centrifuge tube HCHO (5 µL), cHSA-PEO-TPP (1 mg) and Ru1 (1 mg) were mixed in 1 mL pH 5 (PBS buffer) solution. The mixture was vortexed briefly to mix the reaction components, then allowed to shake gently at 37 °C for 72 h. The final solution yielded cHSA-PEO-TPP-Ru in 70% isolated yield and washed several times with water through vivaspin 20 (MWCO 30K) ultracentrifuge to remove excess of Ru1 peptide and storage at 4 °C.

Calculation on reduction of the ABDA absorption peak @380 nm for cHSA-PEO-TPP-Ru and Ru1

We have performed additional head-to-head experiments to show the increased activity of cHSA-PEO-TPP-Ru against Ru1 in reduction of ABDA absorption peak. Firstly, we matched the optical density of Ru of both cHSA-PEO-TPP-Ru and the Ru1 complex. Then, separately both the solution was mixed with ABDA and irradiated with 470 nm LED light source (~20 mW/cm^2, 5 min). We observed a significant decrease in relative absorption of ABDA to 5.05% for cHSA-PEO-TPP-Ru whereas Ru1 showed the decreased to 39.9%. These values collectively indicated ~8-fold increased activity of cHSA-PEO-TPP-Ru and we speculated that this is due to the close proximity of the Ru complexes in the hydrophobic pockets of the protein backbone.

Methods:

Photostability measurements

Samples were dissolved in MilliQ water and irradiated with a 470 nm LED light source for depicted time frames. During irradiation, the samples were stirred and it was ensured that the temperature (rt) was kept constant. The solutions were not degassed to ensure comparability with in vitro studies and with literature.[4]

Two-Photon Absorption Cross Sections measurement

TP action cross section was measured with Rhodamine B in methanol as a reference. The femtosecond (fs) Ti:Sapphire laser (Spectra-Physics Mai Tai® Ti:Sapphire oscillator) was used. TP fluorescence measurements were performed in 10 mm fluorometric quartz cuvettes with 5 µM cHSA-PEO-TPP-Ru in water, 40 µM Ru1 in water and 200 nM Rhodamine B as a reference in Methanol.[5] The experimental fluorescence excitation and detection conditions were kept constant during the measurement of samples and reference in entire scanning wavelengths (700 nm to 920 nm). The TP absorption cross section of the probes was calculated at each wavelength according to eq.

$$\delta_{sample} = \delta_{reference} \frac{\Phi_{(ref)}\, I_{(sample)}\, C_{(ref)}\, \eta_{(sample)}{}^{2}\, P_{(ref)}{}^{2}}{\Phi_{(sample)}\, I_{(ref)}\, C_{(sample)}\, \eta_{(ref)}{}^{2}\, P_{(sample)}{}^{2}}$$

Where I is the integrated fluorescence intensity, C is the concentration, η is the refractive index, Φ is the quantum yield, and P is the incident power on the sample, subscript "ref" stands for reference samples, "sample" stands for samples. The uncertainty in the measured cross sections was about ±10%.

Cell Culture

HeLa cells (human adenocarcinoma cervical epithelial cell line) were cultured in DMEM medium with high glucose supplemented and with 10% fatal bovine serum (FBS), 1% 100 U/mL Penicillin, 1% 0.1 mg/mL Streptomycin, 1% 0.1 mM non-essential amino acids at 37°C in a humidified 5% CO_2 incubator.

The human AML cell line OCI-AML3 was purchased from DSMZ (The Leibniz Institute DSMZ - German Collection of microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and cultured in RPMI 1640 medium with 20% FBS and 1% Penicillin-Streptomycin. The murine AML1-ETO 9a positive AML cell line was established from the bone marrow of a leukemic mouse transduced with a AE9a retroviral construct and cultured in RPMI 1640 medium +20% FBS+1% Penicillin-Streptomycin and IL3. Healthy murine BM cells were isolated from C57BL/6J mice injected with 5-Fluorouracil (150mg/kg, Medac, Germany) to enrich for stem and progenitor cells. Cells were cultured in DMEM medium containing mIL3 (6 ng/mL), mIL6 (10 ng/mL) and mSCF (100 ng/mL).

Cell uptake study

HeLa cells were seeded in densities of 5×10^4 cells per well (if not otherwise stated) in a total volume of 1.5 mL complete culture medium in 24 well-plate. Plates were kept in high glucose DMEM medium at 37 °C for 24 h prior to cHSA-PEO-TPP-Ru addition to ensure that the cells were adherent during the uptake experiment.

After the incubation time (as stated in the experiment) the media from the well were removed and washed three times with 0.5 mL of Dulbecco's PBS to remove non-specific binding, followed by trypsinization with 0.5 mL Trypsin-EDTA per well. The cell number in an aliquot of the resulting suspension was determined in a hemocytometer, and the absolute cell number per

well was calculated. The cell suspension was transferred to tubes for flow-cytometry study. BD Flow Cytometer was used for the measurement of cell uptake using PE channel: Excitation 488 nm & Emission 585/45 nm.

Cellular Uptake Monitored by Laser Scanning Confocal Microscopy

HeLa cells were seeded at a density of 30,000 cells per well in a µ-Slide 8-well chambered coverslip (ibidi, Martinsried, Germany) in 300 µl DMEM medium. The cells were cultured overnight to allow adhesion at 37°C, 5% CO_2. Subsequently, the medium was removed and 500 µM of cHSA-PEO-TPP-Ru was added. The cells were then further incubated for 4 h in the incubator at 37°C, 5% CO2. Before imaging, cells were washed with DMEM medium for 3 times. The live cell imaging was performed using a LSM 710 laser scanning confocal microscope system (Zeiss, Germany) coupled to an XL-LSM 710 S incubator and equipped with a 63x oil immersion objective. The emission of the cHSA-PEO-TPP-Ru was recorded using a 530-710 nm filter and a 458 nm Argon laser for excitation. The acquired images were processed with ZEN 2011 software.

Phototoxicity

HeLa cells were pre-cultured in high glucose DMEM medium with 10% fatal bovine serum, 1% penicillin/streptomycin without phenol-red and seeded at 6,500 cells/well in a white 96-well (half-area) plate. The cells were left to adhere overnight at 37°C, 5% CO_2. The media was removed and different concentrations of cHSA-PEO-TPP-Ru (dissolved in 50 µl DMEM) were added into each well. The treated cells were subsequently incubated for 4 h at 37°C, 5% CO2. After incubation, the cells were washed and irradiated by a 470 nm LED array ($P \sim 20 \pm 3$ mW/cm^2 for 5 min, 6.9 ± 0.9 J/cm^2). The cells were further incubated for 4 h in the dark. As for the dark control assays, the plate was kept in the dark during the whole process. After washing, the cells were treated with Tox-8 reagent. After 2 h incubation, the emission intensity was measured by a Tecan Infinite M1000 microplate reader (λ_{ex} = 570 nm, λ_{em} = 590 nm). The wells without cells but with Tox-8 reagent were used as controls. Each experiment was performed in triplicates. The cell viability rate (VR) was calculated according to the following equation:

$$VR = [(A-C)/(B-C)]\times 100\%$$

Where A is the average emission of experimental groups with the treatment of the compound, B is the average emission of the experimental groups without the treatment of the compound, and C is the average emission of the culture medium background. IC_{50} values for cytotoxicity were calculated by Graph Pad Prism 5.

Culture of AML and normal bone marrow cells

BM cells from healthy mice as well as the AE9a cell line were rapidly thawed, washed twice in RPMI with 20% FCS and incubated with cHSA-PEO-TPP-Ru in tissue culture dishes at a concentration of 6900 cells/mL. After 4h incubation, cells were removed and washed twice in RPMI with 20% FCS. Equal fractions of the cells recovered from cultures with cHSA-PEO-TPP-Ru were exposed to blue light (470nm) for 2min and 5 min, respectively or not exposed to light (dark control). Subsequently, remaining cells were plated into the hematopoietic colony forming cell (CFC) assay. The CFC assay was performed using methylcellulose supplemented with murine cytokines (MethoCult GF M3434, Stem Cell Technologies, Cologne, Germany) as previously described.[6] 7 days after setting up the CFC, number of colonies and morphology of the colonies were assessed. 1000 cells per dish were plated.

Cells from the OCI-AML3 cell line were treated accordingly. Hematopoietic colony forming cell (CFC) assays of human cells were performed using methylcellulose (MethoCult GF H4330, Stem Cell Technologies, Cologne, Germany) as previously described[6] 14 days after setting up the CFC, number of colonies and morphology of the colonies were assessed.

Images of the colonies were acquired using a Nikon eclipse Ti-s inverted microscope and processed using Infini-ty analyze 5.0.3 software (Lumenera corp. Ontario, Cana-da). For proliferation assays 50,000 cells were incubated with the compound for 4 hours, washed and plated. Later on, these cells were either activated for 2 min and 5 min light or left in the dark as a

control. Cell counts were deter-mined using trypan blue exclusion 24 h, 48 h and 72 h after exposure to the drug to assess the effect of the com-pound on the cells. Experiments were performed in biological triplicates (n=3).

Statistical analysis

Data were evaluated using the t test for independent samples. Differences with p values less than 0.05 were considered to be statistically significant. Values mentioned are Mean ± SEM. PRISM GraphPad PRISM® software, Prism 6 for Windows, Version 6.01 (La Jolla, California, USA) was used for the analysis and figures.

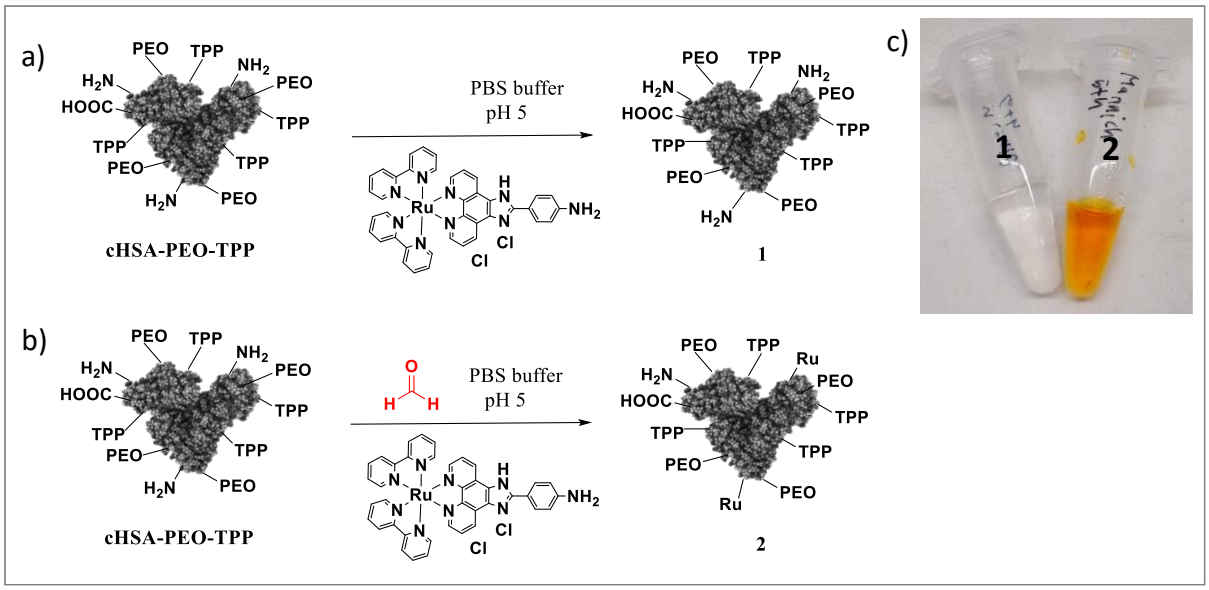


Figure SI-1: a) Ru1 was stirred with cHSA-PEO-TPP bio-hybrid in PBS buffer pH 5 for overnight, **without formaldehyde (HCHO)**. (b) Similar reaction was performed in the **presence of formaldehyde (HCHO).** (c) After purification with dialysis the reaction products **1** and **2** was compared. Without formaldehyde addition, no loading of Ru1 to the cHSA-PEO-TPP bio-hybrid was observed.

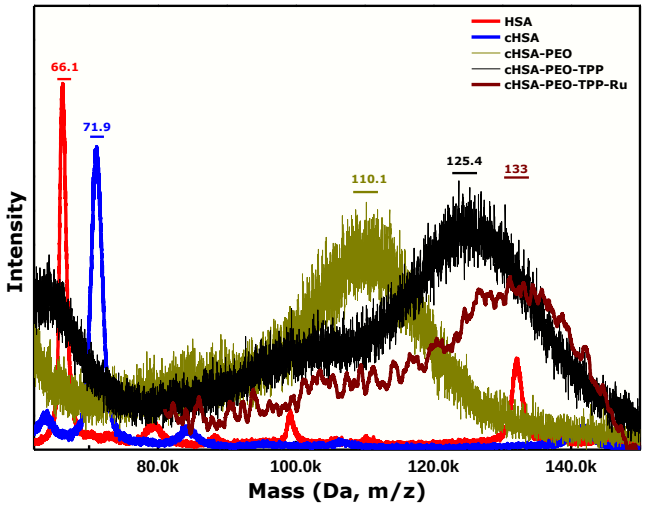


Figure SI-2: (b) MALDI-ToF spectra (matrix: sinapinic acid) indicate successful functionalization from the progressive increase in molecular weight from HSA (calculated 66.4 kDa, measured 66.1 kDa), cHSA (calcu-lated 72.2 kDa, measured 71.9 kDa), cHSA-PEO (calculated 109.8 kDa, measured 110.1 kDa), cHSA-PEO-TPP (calculated 125.5 kDa, measured 125.4 kDa) and cHSA-PEO-TPP-Ru (calculated 133.1 kDa, measured 133 kDa). Approximately 10 Ru-units were attached.

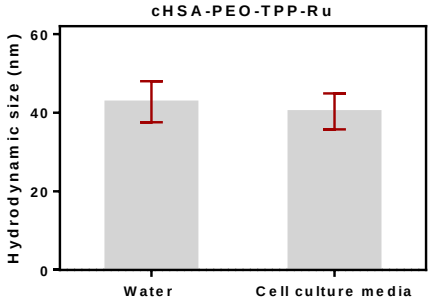


Figure SI-3: Dynamic light scattering values of cHSA-PEO-TPP-Ru conjugates in water and cell culture media. Similar values ~ 40 nm in both cases suggest no larger aggregate formation in cell media.

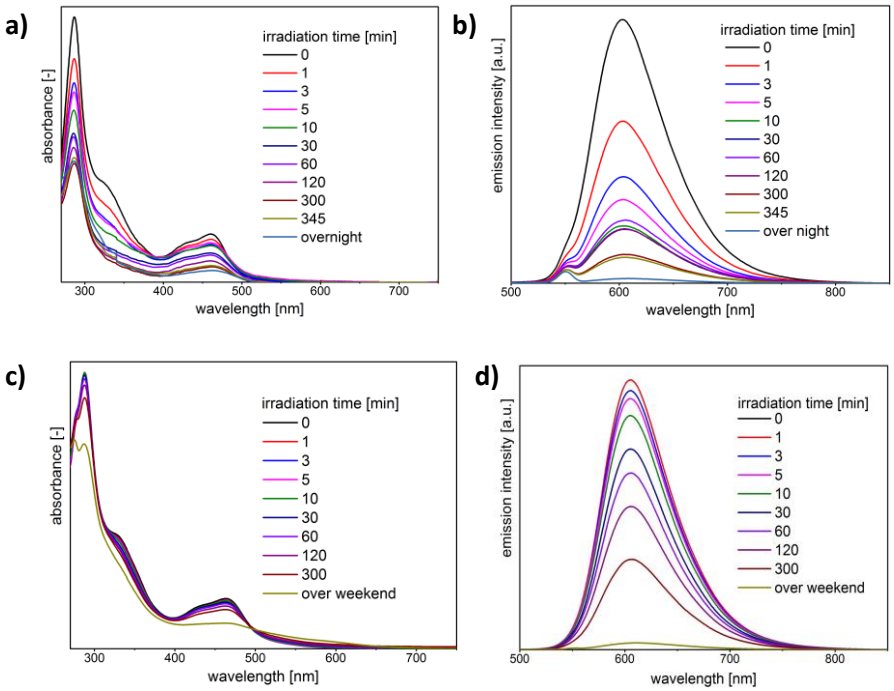


Figure SI-4: Absorbance and luminescence decay under continuous illumination with 470 nm LED light over time, (a-b) Ru1 and (c.d) cHSA-PEO-TPP-Ru in MilliQ water.

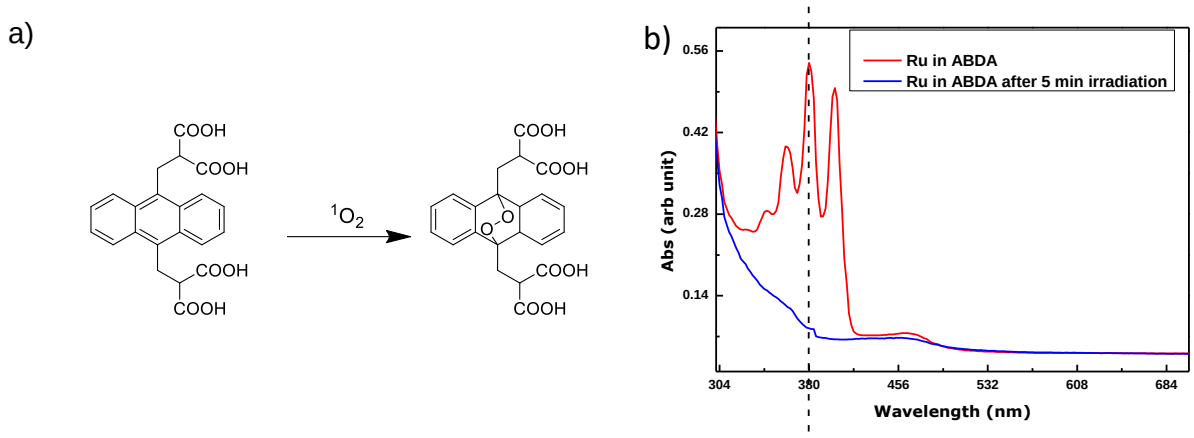


Figure SI-5: a) Scheme showing change in ABDA in the presence of $^1O_2$. b) Absorbance curve of ABDA, Ru1 mixture before and after photo-irradiation using 470 nm LED lamp (~20 mW/cm^2) for 5 min. The reduction in absorbance maxima of ABDA @ 380 nm reflects the singlet oxygen production of Ru1.

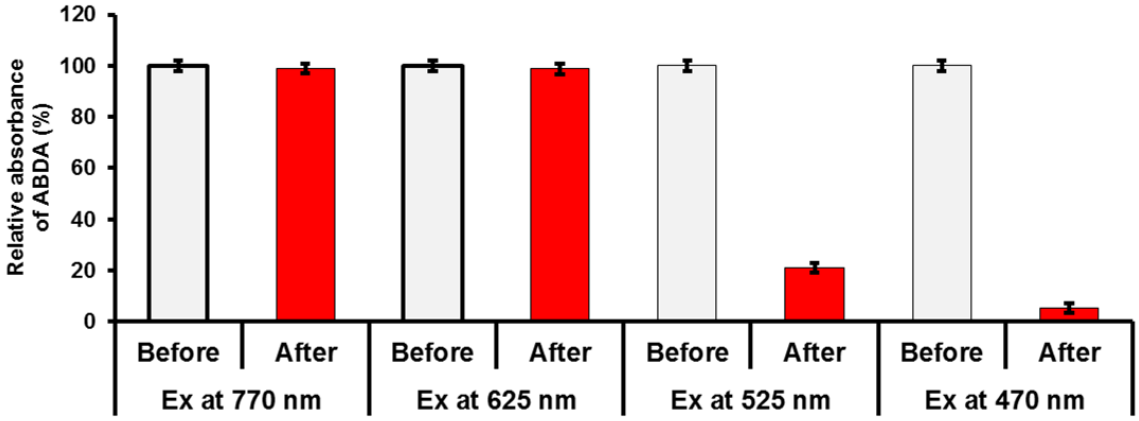


Figure SI-6: Singlet Oxygen production efficiency of cHSA-PEO-TPP-Ru bio-hybrid was measured using the ABDA after 5 min irradiation of each LED light of 470 nm, 525 nm, 625 nm and 770 nm. The bar diagram shows that, among the four LED lights tested the 470 nm light has maximum singlet oxygen production followed by 525 nm LED. Whereas the 625 nm and 770 nm LED lights have negligible singlet oxygen production. Sample without light irradiation was considered control (100% ABDA absorbance).

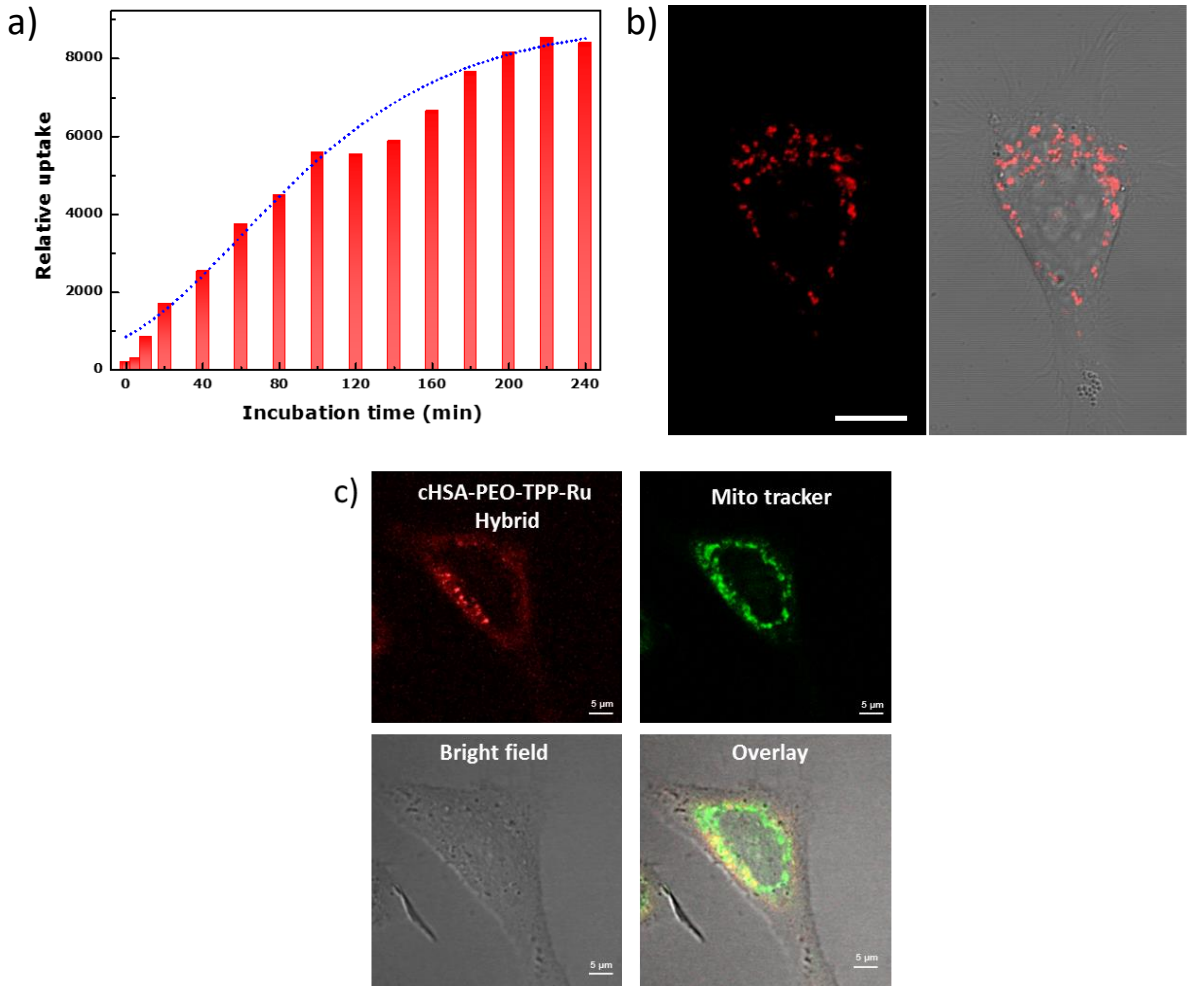


Figure SI-7: a) Cell uptake of Protein-Ru Hybrid (500 nM) into HeLa cells in a time-dependent fashion until saturation is reached. HeLa cells were incubated with 500 nM Protein-Ru Hybrid for various time intervals ranging from 1 min to 240 min (4 h) and analysed by BD Flow-cytometry. b) Confocal imaging of HeLa cells incubated with 500 nM Protein-Ru Hybrid for 4 h at 37 °C. Scale bare 10 µm. (c) Confocal microscopy images of HeLa cells incubated with cHSA-PEO-TPP-Ru and treated with commercial mitochondria trackers. Overlay images and colocalization analysis of cells proved efficient mitochondria colocalization.

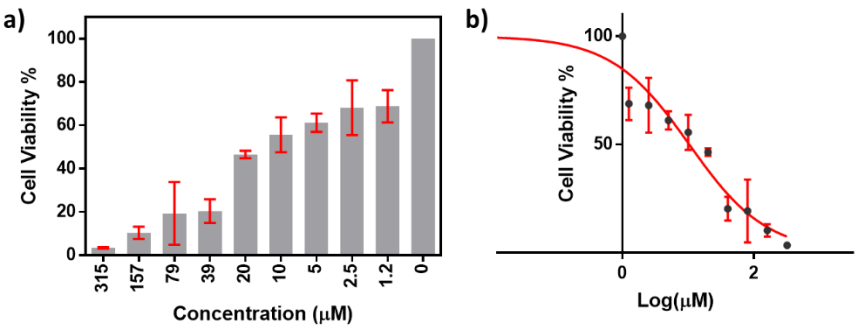


Figure SI-8: (a) Cell viability study of Ru1 tested within a broad concentration range from 1.2 µM to 315 µM, where HeLa cells were incubated with Ru1 and thereafter exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability assay. (b) The logarithmic fitting curve for cell viability of Ru1. IC_{50} value of Ru1 is 7.7± 1.3 µM.

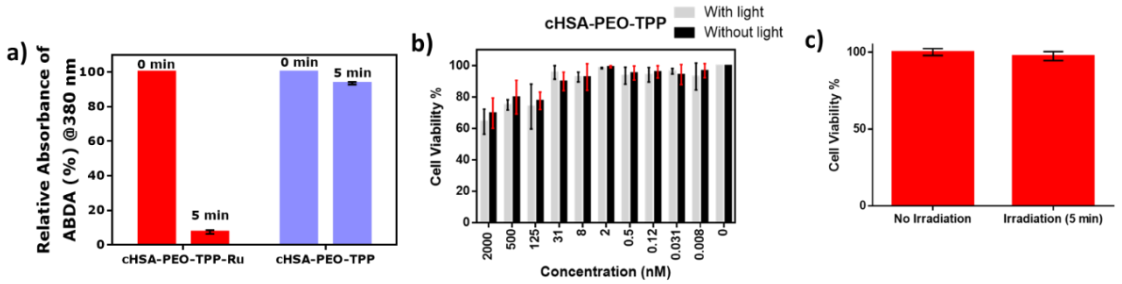


Figure SI-9: (a) $^1O_2$ production yield of cHSA-PEO-TPP-Ru and cHSA-PEO-TPP, as obtained from the photobleaching of characteristics @380 nm absorption peak of ABDA (100 µM) during irradiation with 470 nm LED light (~20 mW/cm^2, 5 min) in PBS (1x, pH 7.4). (b) Cell viability study of cHSA-PEO-TPP in a broad concentration range from 0 nM to 2 µM with and without light, where HeLa cells were incubated with cHSA-PEO-TPP and thereafter exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability assay. cHSA-PEO-TPP shows no significant photo-toxicity. (c) HeLa cells were exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability was measured. Cells that were not exposed to light were used as control. The application of light alone had no effect on cell viability under these experimental conditions.

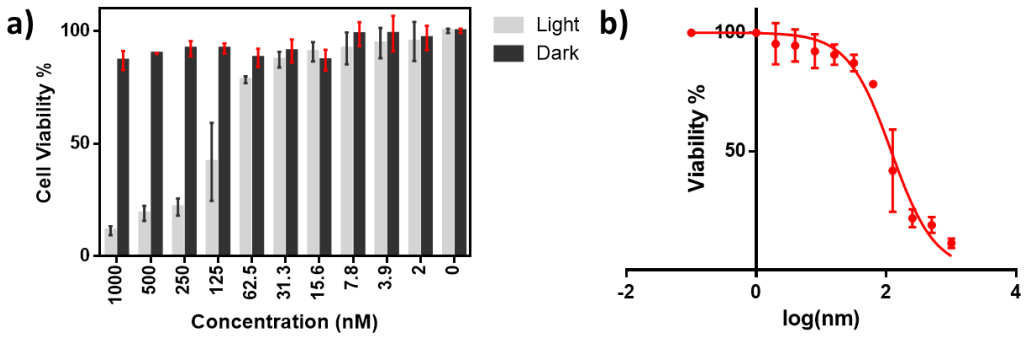


Figure SI-10: Cell viability study of cHSA-PEO-TPP-Ru using **CHO cells**. (a) CHO cells incubated with cHSA-PEO-TPP-Ru in broad concentration range (0 nM to 1000 nM), and were exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability assay. Cells without light exposure were also measured for their dark toxicity. (b) The logarithmic fitting curve for cell viability using CHO cells. IC_{50} value of of cHSA-PEO-TPP-Ru for CHO cells is 135.2 ± 1 nM.

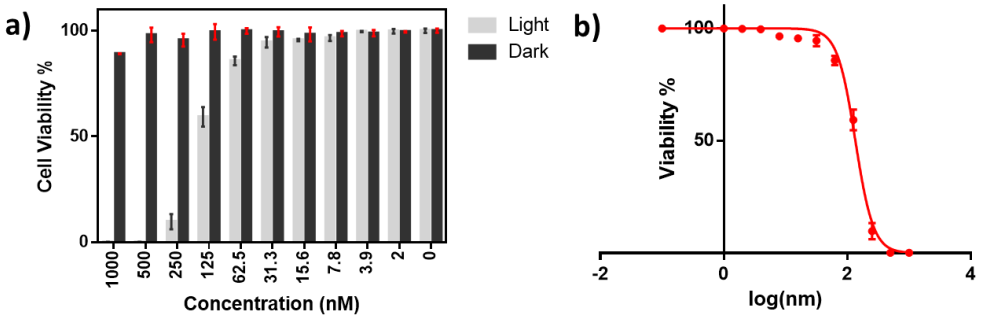


Figure SI-11: Cell viability study of cHSA-PEO-TPP-Ru using **MCF7 cells**. (a) MCF7 cells incubated with cHSA-PEO-TPP-Ru in broad concentration range (0 nM to 1000 nM), and were exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability assay. Cells without light exposure also measured for dark toxicity. (b) The logarithmic fitting curve for cell viability using MCF7 cells. IC_{50} value of of cHSA-PEO-TPP-Ru for MCF7 cells is 114.3 ± 1 nM.

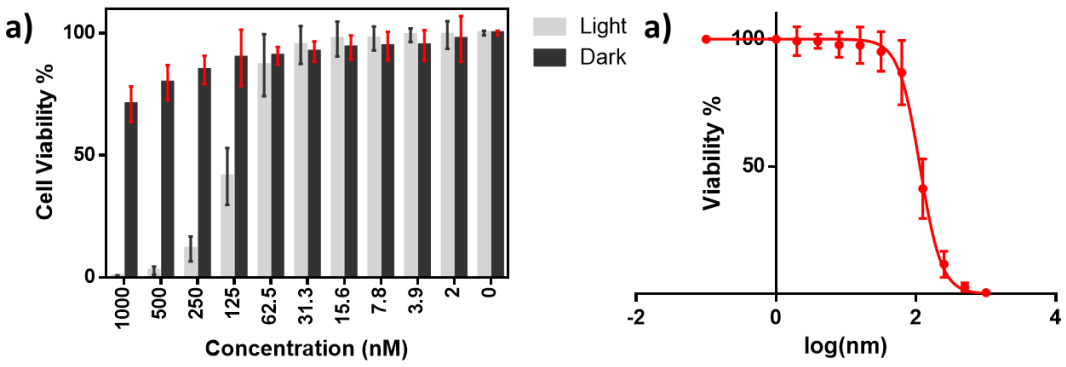


Figure SI-12: Cell viability study of cHSA-PEO-TPP-Ru using **A549 cells**. (a) A549 cells incubated with cHSA-PEO-TPP-Ru in broad concentration range (0 nM to 1000 nM), and were exposed to 470 nm LED lamp (~20 mW/cm^2) for 5 min before cell viability assay. Cells without light exposure also measured for dark toxicity. (b) The logarithmic fitting curve for cell viability using A549 cells. IC_{50} value of of cHSA-PEO-TPP-Ru for A549 cells is 119.1 ± 1 nM.

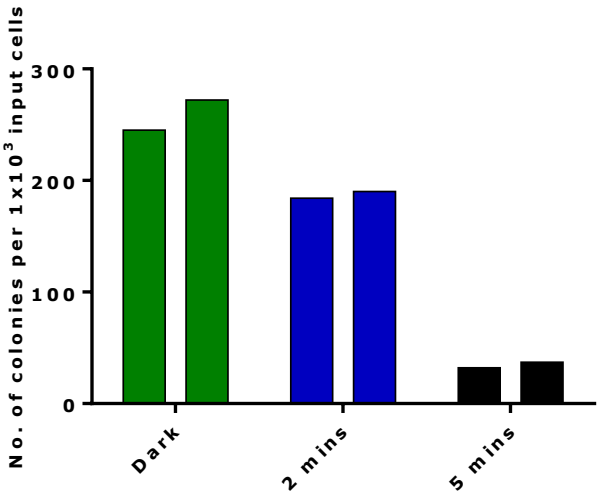


Figure SI-13: Colony forming cell (CFC) assay of AML1-ETO9a cell lines treated with cHSA-PEO-TPP-Ru showed a decrease in colony numbers after irradiating cells for 2 min and 5 min compared to the cells treated but not irradiated (dark) (n=1; duplicates).

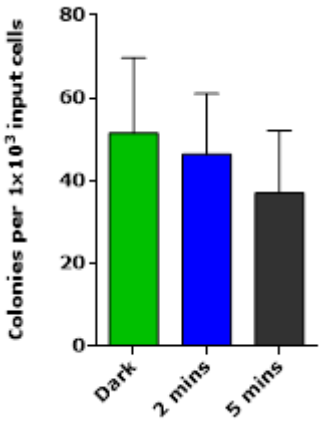
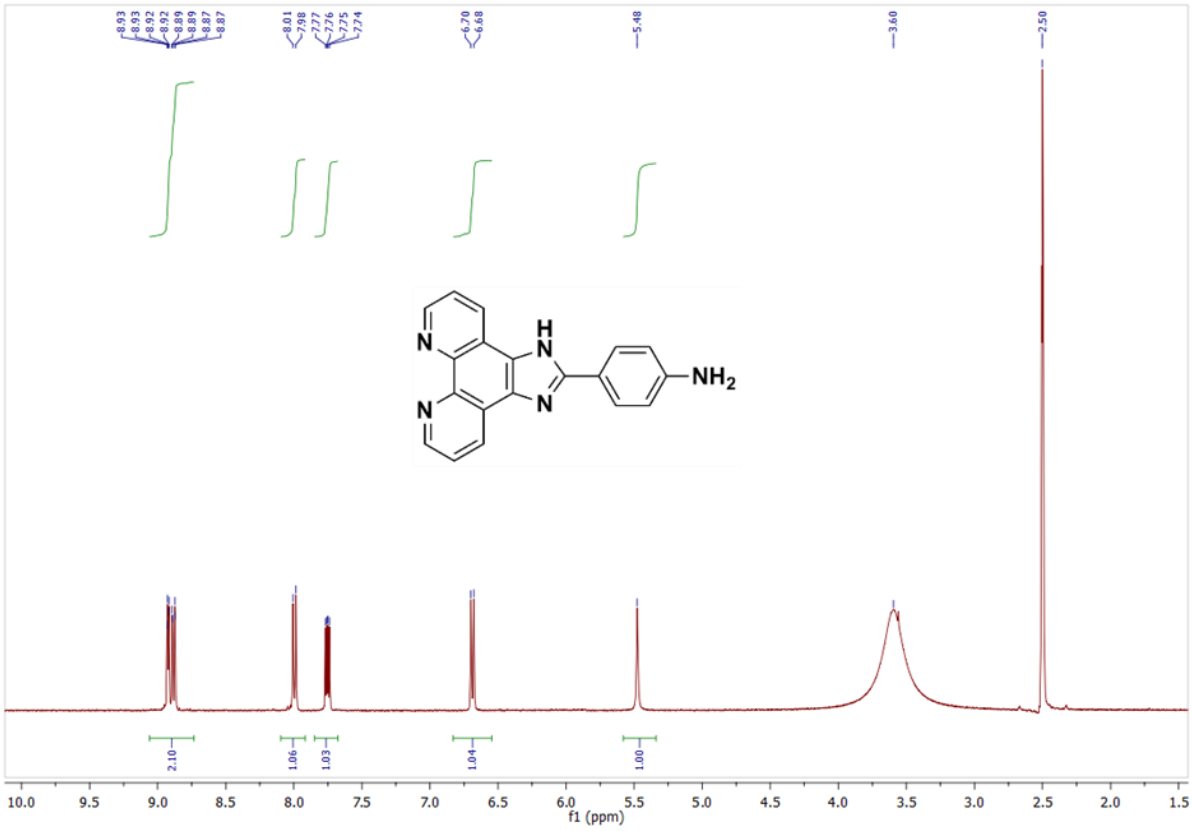
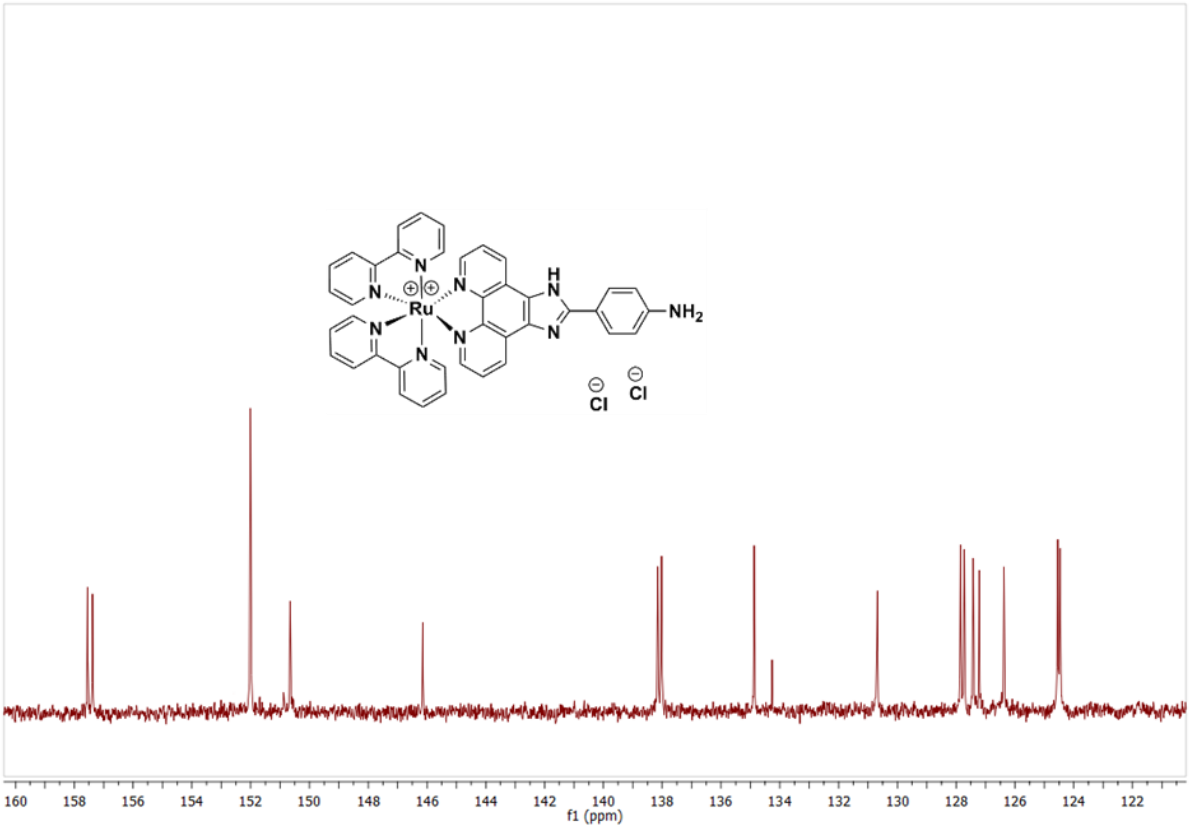


Figure SI-14: Colony forming cell (CFC) assay of normal bone marrow (nBM) treated with cHSA-PEO-TPP-Ru did not show a significant decrease in colony number after irradiating cells for 2 min or even 5 min; compared to the cells treated but not irradiated (dark) (n=3; duplicates). Bars show Mean±SEM.

NMR Data:





Composition simulated body fluid:

SBF – Updated Simulated Body Fluid pH = 7,4			
Substance	Mass (gm)		Charge
$CaCl_2$ * 2 H_2O	0,387		430155401
KCl	0,225		29087021
KH_2PO_4	0,138		140155750
$MgCl_2$	0,146		035223846
NaCl	8,035		K41420604037
$NaHCO_3$	0,355		81128
$NaSO_4$	0,072		TA273837
TRIS	6,118		280159067
1 M HCl	39 mL		08J030508 (37 %)

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