Rationally designed ruthenium complexes for 1- and 2-photon photodynamic therapy

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The use of photodynamic therapy (PDT) against cancer has received increasing attention over recent years. However, the application of the currently approved photosensitizers (PSs) is limited by their poor aqueous solubility, aggregation, photobleaching and slow clearance from the body. To overcome these limitations, there is a need for the development of new classes of PSs with ruthenium(II) polypyridine complexes currently gaining momentum. However, these compounds generally lack significant absorption in the biological spectral window, limiting their application to treat deep-seated or large tumors. To overcome this drawback, ruthenium(II) polypyridine complexes designed in silico with \((\text{E,E'})\)-4,4'-bisstyryl-2,2'-bipyridine ligands show impressive 1- and 2-Photon absorption up to a magnitude higher than the ones published so far. While nontoxic in the dark, these compounds are phototoxic in various 2D monolayer cells, 3D multicellular tumor spheroids and are able to eradicate a multiresistant tumor inside a mouse model upon clinically relevant 1-Photon and 2-Photon excitation.

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Due to the increasing impact of cancer on the life quality and mortality of humans, increasing research efforts are devoted toward the development of anticancer drugs and strategies. Among the most commonly used techniques to fight this disease (i.e., surgery, chemotherapy, and radiotherapy), photodynamic therapy (PDT) is receiving increasing attention during the last decades. In PDT, a photosensitizer (PS) is activated upon light irradiation to generate reactive oxygen species (ROS). As the majority of currently approved PSs are based on tetrapyrrolic structures (i.e., porphyrin, chlorin, and phthalocyanine), these compounds share similar drawbacks (e.g., poor aqueous solubility, aggregation, photobleaching, slow clearance from the body, and hepatotoxicity). To overcome these limitations, there is a need for the development of new classes of PSs. Among others, the use of transition metal complexes and especially, Ru(II) polypyridine complexes are gaining momentum due to their attractive photophysical and chemical properties (i.e., strong luminescence, high singlet oxygen production, high chemical, and photophysical stability). With the compound TLD-1433 having just entered phase II clinical trials for the treatment of non-muscle invasive bladder cancer, despite these remarkable properties, the vast majority of Ru(II) polypyridine complexes are excited using either blue or UV-A light. As the light tissue penetration depth is rather poor at these wavelengths, the application of these compounds to treat deep-seated or large tumors is limited. To circumvent this drawback, there is a need for the development of PSs with an absorption towards the biological spectral window (600–900 nm), which can be achieved by a red-shifted one-photon (1P) absorption or the use of a two-photon (2P) absorption process for 2P PDT, a technique that is not employed yet in the clinic. As trans-stilbene are highly effective 2P dyes, it would be of high interest to extend the ligand scaffold with such a moiety as well as to include terminal donor groups, namely bis[4,4′-stilbene is highly effective 2P dyes but also from ligand-centered (LC) charge-transfer excitations. Detailed analysis of the 1P and of the main bands in the 400–900 nm region, stemming from both MLCT/LMCT type transitions but also from ligand-centered (LC) charge-transfer excitations. Detailed analysis of the 1P and of the lowest lying 2P computed data (Supplementary Tables 1–4) indicated that the most intense lowest lying 2P absorption processes are associated with LCCT transitions with an average CT distance of 2.9–6.3 Å. Overall, the computed data suggests that Ru(II) coordinated (E,E′)-4,4′-bis-styryl-2,2′-bipyridine complexes with donor substituents, have a red-shifted 1P and a strong 2P absorption.

**Results**

**Rational design.** With the aim of enhancing the absorption properties of Ru(2,2′-bipyridine), without deterioration of its 1P absorption properties, the bipyridine ligand was functionalized with rigid π conjugated substituents acting as electron-donating groups. This is expected to induce a 1P absorption red-shift toward the biological spectral window by intercalation of donor-centered orbitals in the Ru centered frontier orbitals manifold (allowing transitions of metal-to-ligand charge transfer (MLCT) and ligand-to-metal charge transfer (LMCT) character at low energy). As trans-stilbene are highly effective 2P dyes, it would be of high interest to extend the ligand scaffold with such a moiety as well as to include terminal donor groups, namely bis[4,4′-stilbene is highly effective 2P dyes but also from ligand-centered (LC) charge-transfer excitations. Detailed analysis of the 1P and of the lowest lying 2P computed data (Supplementary Tables 1–4) indicated that the most intense lowest lying 2P absorption processes are associated with LCCT transitions with an average CT distance of 2.9–6.3 Å. Overall, the computed data suggests that Ru(II) coordinated (E,E′)-4,4′-bis-styryl-2,2′-bipyridine complexes with donor substituents, have a red-shifted 1P and a strong 2P absorption.

**Synthesis and characterization.** Before the synthesis of the final compounds (Fig. 1), the experimental procedures to obtain the corresponding ligands were optimized to a one-step high-yielding synthesis using mild mild conditions (Supplementary Fig. 2).
Having the ligands in hand, the desired complexes 1–7 were synthesized. All compounds were analyzed by $^1$H, $^{13}$C-NMR, ESI-HRMS (Supplementary Figs. 3–25) and their purity confirmed by HPLC as well as elemental analysis. In addition, the ligands L-H, L-NMe$_2$, and L-OMe as well as 3 were characterized using single-crystal X-ray crystallography (Supplementary Tables 5–6, Supplementary Figs. 26–29).

Photophysical evaluation. The photophysical properties of the complexes (Table 1, Supplementary Fig. 30) were then experimentally investigated to evaluate their potential as PDT PSs. In agreement with theoretical findings, the compounds generally show a red-shift of the first 1P absorption (Fig. 2a) band maximum of about 50–70 nm for the symmetric Ru(II) complexes, in comparison to Ru(2,2'-bipyridine)$_2$ as well as an absorption tail toward the near-infrared region. Strikingly and in agreement with theoretical data, the reported compounds have an exceptionally strong 2P absorption (Fig. 2b) with values up to ~6800 GM, which is an order of magnitude higher than the ones reported before for other Ru(II) polypyridine complexes $^{32-38}$. Interestingly, the L-NMe$_2$ coordinated complexes were found with larger $\sigma_2$ values than the L-OMe and L-H coordinated compounds. Overall, the compounds have a 1P absorption tail towards, and a strong 2P absorption, in the biological spectral window, potentially allowing for the treatment of deep-seated or large tumors.

The luminescence quantum yields of the L-OMe coordinated complexes (3: 1.1%, 5: 1.4%, 7: 2.8%) were found to be significantly higher than those of the L-H coordinated (1: 1.9%) or L-NMe$_2$ coordinated (2: >0.1%, 4: 0.4%, 6: 0.5%) compounds. All complexes were found to have excited state lifetimes in the nanosecond range (Supplementary Figs. 31–37) in degassed (222–542 ns) and aerated saturated (36–96 ns) solutions. As the lifetimes drastically decrease in the presence of air, it indicates that the excited state can interact with a component in the air. For identification of the type of ROS produced upon light exposure, electron spin resonance (ESR) spectroscopy was employed using the singlet oxygen ($^1$O$_2$) scavenger 2,2,6,6-tetramethylpiperidine and the *OOH or *OH radical scavenger 5,5-dimethyl-1-pyrroline N-oxide. While no signals for the formation of *OOH or *OH radicals were detected, the formation of $^1$O$_2$ in acetonitrile and PBS was confirmed by observation of the characteristic $^1$O$_2$-induced triplet signal in the ESR spectrum (Supplementary Figs. 38–44). The amount of generated $^1$O$_2$ was quantitatively determined by two methods: directly by measurement of the phosphorescence of $^1$O$_2$; indirectly by monitoring the change in absorbance of a $^1$O$_2$ scavenger. The singlet oxygen quantum yields were found to be between 16–77% in acetonitrile and 1–11% in an aqueous solution (Table 1). The comparison between the values indicates that the L-OMe coordinated complexes (3, 5, 7) are able to produce $^1$O$_2$ more efficiently than 1 and the L-NMe$_2$ coordinated Ru(II) complexes (2, 4, 6). Overall, complex 7 was found with the highest singlet oxygen quantum yield (acetonitrile: 68–77%, aqueous solution: 10–11%).

Stability. The stability of the compounds was investigated by incubation in human plasma at 37 °C for 48 h. The comparison of the HPLC chromatograms (Supplementary Figs. 45–51) showed no change before and after incubation in human plasma for all compounds, indicative of the stability of the complexes under biological conditions. Additionally, the stability upon irradiation was investigated (Supplementary Figs. 52–60) as the majority of current clinically employed PSs suffer from this drawback. Importantly, no significant differences in the absorption spectra for the L-OMe coordinated complexes (3, 5, 7) and 1 were observed over time, indicating their photostability. On the
contrary, small changes in the absorption spectra of the L-NMe3 coordinated complexes (2, 4, 6), especially of 2, were observed, suggesting that the complexes are slightly photobleaching. Worthy of note, under identical experimental conditions, the absorption spectra of the known PS Protoporphyrin IX (PpIX) was found to be drastically changed, indicating a significantly stronger photobleaching. The study of the effect of the irradiation on the molecular structure of 2 by NMR spectroscopy suggests the decomposition of the compound (Supplementary Fig. 61).

Biological evaluation on 2D monolayer cells. All compounds were found to be lipophilic with high distribution coefficients between an organic octanol and an aqueous PBS phase (Supplementary Table 7). The time-dependent cellular uptake of the complexes was then investigated in human cervical carcinoma (HeLa) cells by determining the amount of Ru inside the cells by inductively coupled plasma mass spectrometry (ICP-MS) at each time point. The results show that the asymptotic maximum (Supplementary Figs. 62–68) of the uptake of the compounds was reached within 8 h. As expected, the compounds with a higher lipophilicity, with the exception of 2, were found to have the highest uptake (Supplementary Fig. 69). The uptake mechanism of the complexes was then investigated by blocking various pathways by preincubation with a cationic transporter (tetraethylammonium chloride), metabolic (2-deoxy-D-glucose and oligomycin), and endocytic inhibitors (ammonium chloride or oligomycin), and phototoxic (cycloheximide) and necrotic (necrostatin-1) inhibitors (Supplementary Fig. 82). While apoptosis was found to be the cell death mechanism for 1–5, 6–7 triggered cell death by a combination of apoptosis and paraptosis pathways.

Biological evaluation on 3D multicellular tumor spheroids. After evaluation of the biological effects of the compounds on 2D monolayer cells, their ability to act on 3D MCTS was investigated. MCTS simulate the conditions found in clinically treated tumors including hypoxia and proliferation gradients to the center. Consequently, the penetration of the compounds inside of HeLa MCTS with a diameter of 800 μm was investigated by 1P and 2P z-stack confocal laser scanning microscopy. 4–7 completely penetrated the MCTS within 12 h with a strong luminescence signal at every section depth, whereas 1–3 were mostly found on the outer sphere (Supplementary Figs. 83–89). Upon an increased incubation time up to 60 h, the remaining compounds were also able to penetrate the MCTS, with the exception of 2, which was still found mostly on the outer sphere (Supplementary Figs. 90–94). Following this, the tumor growth inhibition effect of
1–7 (20 μM) in HeLa MCTS was investigated and compared with the well-known PS tetraphenylporphyrin (H2TPP) (20 μM) and cisplatin (10 μM, 30 μM). The compounds were incubated for 3 days in the dark as this time was shown to be required for complete MCTS penetration. The MCTS were then exposed to 1P (500 nm, 16.7 min, 10.0 mW cm−2, and 10 J cm−2) or 2P irradiation (800 nm, 10 J cm−2, and section interval of 5 μm) on day 3. During the whole time period, the shape and volume of the MCTS was constantly monitored. As expected, the MCTS treated with 1–7 or H2TPP in the dark (Fig. 3a, representative image of MCTS: Supplementary Fig. 95) were asymptotically growing in the same manner than the control group, indicating that the compounds do not show any inhibitory effect, whereas cisplatin showed a weak effect on the tumor growth. On the contrary, the volume of the MCTS treated with complexes 1–7 and exposed to 1P or 2P irradiation (Fig. 3b, c, representative image of MCTS: Supplementary Figs. 96–97) significantly shrank, demonstrating their strong tumor inhibition effect.

To further study the effect the complexes have on the tumor survival, the treated MCTS were stained with a cellular living cell kit using Calcein AM (Fig. 4). The fluorescence images confirmed that the MCTS treated with 1–7 in the dark and with 1–3 upon a 1P irradiation (500 nm, 16.7 min, 10.0 mW cm−2, and 10 J cm−2) or 2P irradiation (800 nm, 10 J cm−2, and section interval of 5 μm) are still intact. The low phototoxic effect of 1–3 is caused by the poor MCTS penetration. Promisingly, the MCTS treated with 4–7 and exposed to 1P or 2P light were completely eradicated.

The photodynamic effect using a 1P (500 nm, 16.7 min, 10.0 mW cm−2, and 10 J cm−2) and 2P irradiation (800 nm, 10 J cm−2, and section interval of 5 μm) was then quantified by...
Biological evaluation inside a mouse model. Capitalizing on the superior photophysical (i.e., highest $^{1}$O$_{2}$ production) and biological properties (i.e., highest phototoxicity in 2D monolayer cells and 3D MCTS, high cell penetration), the properties of 7 to act as a PDT PS were further investigated in a mouse model. To ensure solubility and biocompatibility, the PS was converted to a chloride counter ion. We note at this stage that a change of the counter ion can have a significant effect on the biological properties of a metal complex. It was previously shown that a counter ion can cause an undesired cytotoxicity or the formation of nanoggregates or a colloid due to poor water solubility. It was also reported that the cellular uptake of a Ru(II) complex can dramatically change. A challenging to treat multiresistant doxorubicin-selected P-gp-overexpressing human colon cancer tumor model (SW620/AD300) was used to evaluate the PS potential. The biodistribution of 7 (2 mg kg$^{-1}$) inside this mouse model was then time-dependent (30 min, 1 h, 2 h) studied by intravenous tail injection into nude mice. After each time point, the mice were sacrificed and the major organs (i.e., blood, spleen, intestine, stomach, liver, kidney, uterus, lung, heart, brain, and tumor) were separated, ground and the Ru content determined by ICP-MS. Interestingly, 7 was absorbed from the blood stream within 1 h with a high accumulation in the intestine and some accumulation in the tumor (Supplementary Fig. 98). Following this, 1 h after an intravenous tail injection of 7 (2 mg kg$^{-1}$), in vivo PDT experiments using a 1P (500 nm, 60 min, 10 mW cm$^{-2}$, and 36 $\mu$J cm$^{-2}$) or 2P irradiation (800 nm, 50 mW, 1 kHz, pulse width 35 fs, and 5 $s$ mm$^{-1}$) were performed on mice with an 80 mm$^{3}$ tumor. Encouragingly, after only one PDT treatment, the tumor drastically shrank until they were nearly eradicated whereas the tumors treated with the light or tumor (Fig. 5a, c) kept growing. Importantly, the animals treated with the compound behaved normally, without signs of pain, stress or discomfort and did not lose or gain weight (Fig. 5b). After the treatment, the mice were sacrificed, the tumor and organs were separated and histo logically examined by an H&E stain. The tumor tissue treated with 7 and exposed to light displayed pathological alterations caused by the PDT treatment (Supplementary Fig. 99), while all other organs did not show any significant effect (Supplementary Fig. 100). Overall, this study demonstrates the enormous potential of 7 as a PS for 1P and 2P PDT.

In summary, Ru(II) polypyrrole complexes with $(E,E)^{-}\text{Bisstyryl-2,2'}$-bipyridine ligands have been rationally designed using DFT calculations and were found to have a remarkable red-shifted 1P absorption as well as exceptionally high 2P cross sections. The complexes were found to be taken up by cancerous cells through an energy-dependent endocytosis pathway and accumulate in the cytoplasm. Upon irradiation, they were found to generate $^{1}$O$_{2}$, causing a phototoxic effect by apoptosis and paraptosis pathways in various monolayer cells as well as MCTS. In vivo studies confirmed the impressive ability of 7 to act as a PS upon treatment at clinically relevant 1P (500 nm) and 2P (800 nm) irradiation with an eradication of the tumor. Importantly, these compounds were found to be highly water soluble, stable in human plasma, as well as upon constant irradiation and therefore are promising for the limitation of currently employed PSs. We strongly believe that these complexes have great potential for preclinical trials.

**Methods**

**Computational details.** All calculations were performed at DFT (ground state) and TD-DFT (excited state) level using the global hybrid B3LYP functional. The Los Alamos (LANL2) effective core potential and the corresponding triple-zeta basis set was applied to describe the Ruthenium atom, with all other atoms were described by a Pople double-zeta basis set with a single set of polarization functions on non-hydrogen atoms ($6-31G(d)$). All structures used correspond to ground state minima on the ground state potential energy surfaces. Solvent effect (acet onitrile) were taken into account using the PCM model for structural optimizations while single point calculations in the gas phase were performed to simulate 1P and 2P absorption spectra. All calculations were performed using the Gaussian suite of programs except for the 2P spectra which were simulated using the DALTON software.

**Materials.** All chemicals were purchased from commercial sources and used without further purification. If necessary, solvents were dried over molecular sieves. The Ru(II) precursors Ru(DMSO)$_2$Cl$_2$ and Ru(2,2'-bipyridine)$_2$Cl$_2$ were synthesized according to previously reported procedures. The pooled human plasma was obtained from Biowest. The cell culture media and reagents were purchased from Fisher Scientific.

**Instrumentation and methods.** $^1$H and $^{13}$C-NMR spectra were measured on a Bruker 400 MHz or 500 MHz NMR spectrometer. Chemical shifts ($\delta$) are reported in parts per million (ppm) referenced to tetramethylsilane ($\delta$ 0.00 ppm) using the residual proton solvent peaks as internal standards and coupling constants ($J$) in Hertz (Hz). The multiplicity of the peaks is abbreviated as follows: s (singlet), d (doublet), t (triplet), m (multiplet). ESI-MS experiments were carried out using a LTQ-Orbitrap XL from Thermo Scientific and operated in positive ionization mode with a spray voltage of 3.6 kV. No Sheath and auxiliary gas was used. Applied voltages were 40 and 100 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275 °C. Detection was achieved in the Orbitrap with a resolution set to 100,000 for 1P and 2P absorptions spectra. All calculations were performed using the Gaussian suite of programs except for the 2P spectra which were simulated using the DALTON software.

**Synthesis.** $(E,E)^{-}\text{4,4'}\text{-Bisstyryl-2,2'}$-bipyridine: The synthesis of $(E,E)^{-}\text{4,4'}\text{-Bisstyryl-2,2'}$-bipyridine is already published but in this study another synthetic route was employed. 4,4'-Dimethyl-2,2'-bipyridine (1000 mg, 5.43 mmol, 1.0 equiv.) was dissolved in dry N,N-dimethylformamide under nitrogen atmosphere and benzaldehyde (1.2 mL, 11.84 mmol, 2.2 equiv.) was added to the solution. Afterwards paraformaldehyde (2436 mg, 72.72 mmol, 4.0 equiv.) was added slowly. The color of the solution turned to green and the mixture was stirred for 24 h. After that the mixture was poured into water (400 mL) and the suspension cooled down to 5 °C. The crude product which precipitated, was filtered and washed with methanol. The product was purified by recrystallization from boiling acetic acid. The obtained solid was dissolved in dichloromethane and the mixture was washed with a 5% aqueous lithium chloride solution, brine and water. The solvent was removed and the product was isolated by recrystallization from boiling acetic acid. 1292 mg of $(E,E)^{-}\text{4,4'}\text{-Bisstyryl-2,2'}$-bipyridine (3.58 mmol, 66%) were yielded as a beige solid. The experimental data obtained is in agreement with the previous literature. $(E,E)^{-}\text{4,4'}\text{-Bis[p(N,N-dimethylamino)styryl]-2,2'}$-bipyridine: The synthesis of $(E,E)^{-}\text{4,4'}\text{-Bis[p(N,N-dimethylamino)styryl]-2,2'}$-bipyridine is already published but
in this study another synthetic route was employed. 4,4′-Dimethyl-2,2′-bipyridine (1000 mg, 5.43 mmol, 1.0 equiv.) was dissolved in dry N,N-dimethylformamide (100 mL) under nitrogen atmosphere and potassium tert-butoxide (2437 mg, 21.72 mmol, 4.0 equiv.) was added slowly. After 1.5 h of stirring, 4-(dimethylamino)benzaldehyde (1701 mg, 11.40 mmol, 2.1 equiv.) was added to the reaction mixture. The color of the solution turned to yellow and the mixture was heated at 90 °C for 19 h. After that the mixture was poured into water (400 mL) and the suspension cooled down to 5 °C. The crude product which precipitated, was filtered and washed with water and diethyl ether. The product was isolated by recrystallization from dichloromethane/pentane. 1541 mg of (E,E′)-4,4′-Bis[p-(N,N-dimethylamino)styryl]-2,2′-bipyridine (3.45 mmol, 64%) were yielded as a yellow solid. The experimental data obtained is in agreement with the previous literature.

(E,E′)-4,4′-Bis[p-methoxy styryl]-2,2′-bipyridine: The synthesis of (E,E′)-4,4′-Bis [p-methoxystyrlyl]-2,2′-bipyridine is already published but in this study another synthetic route was employed. 4,4′-Dimethyl-2,2′-bipyridine (532 mg, 2.89 mmol, 1.0 equiv.) was dissolved in dry N,N-dimethylformamide (25 mL) under nitrogen atmosphere and 4-methoxybenzaldehyde (0.88 mL, 7.22 mmol, 4.0 equiv.) was added to the solution. Afterwads potassium tert-butoxide (1360 mg, 12.13 mmol, 4.2 equiv.) was added slowly. The color of the solution turned to green and the mixture was stirred for 24 h. After that the mixture which turned bright was poured into water (200 mL) and the suspension cooled down to 5 °C. The crude product which precipitated, was filtered, and washed with methanol. The product was purified by recrystallization from boiling acetic acid. The obtained solid was dissolved in dichloromethane and the mixture was washed with a 5% aqueous lithium chloride solution, brine and water. The solvent was removed and the product was isolated by recrystallization from boiling acetic acid. 925 mg of (E,E′)-4,4′-Bis[p-methoxystyryl]-2,2′-bipyridine (2.20 mmol, 76%) were yielded as a beige solid. The experimental data obtained is in agreement with the previous literature.

[Ru(E,E′)-4,4′-Bisstyryl-2,2′-bipyridine)$_2$][PF$_6$]$_2$ (1): (E,E′)-4,4′-Bisstyryl-2,2′-bipyridine (400 mg, 1.11 mmol, 4.0 equiv.) and Ru(DMSO)$_4$Cl$_2$ (134 mg, 0.28 mmol, 1.0 equiv.) were suspended in dry ethanol (150 mL) under nitrogen atmosphere and the mixture was refluxed for 24 h. Then the solution was cooled down and undissolved residue was removed by filtration. To the residual solution a
saturated aqueous solution of ammonium hexafluoroarsenate was added. The crude product, which precipitated as a hexafluoroarsenate salt was collected by centrifugation and washed with ethanol, water and diethyl ether. The product was dissolved in dichloromethane and washed with 5% aqueous lithium chloride solution, brine and water. After drying, 323 mg of 1 (0.02 mmol, 79%) were yielded as a red solid. 1H-NMR (500 MHz, CDCl3): δ 8.76 (d, J = 1.8 Hz, 6H), 7.78 (d, J = 6.6 Hz, 6H), 7.67 (d, J = 6.9 Hz, 6H), 7.34 (d, J = 8.2 Hz, 6H), 7.24–7.09 (m, 12H), 7.03 (d, J = 1.8 Hz, 6H), 6.74 (d, J = 16.2 Hz, 1H), 7.64 (d, J = 15.8 Hz, 4H, 2H), 7.43–7.34 (m, 6H), 7.02 (d, J = 16.2 Hz, 4H), 6.81–6.77 (m, 8H), 3.02 (s, 12H), 3.01 (s, 12H), 1.31 (s, 36H), 1.17 (d, J = 7.1 Hz, 6H), 0.89 (s, 3H) (Found for C84H72F12N6O6P2Ru): C (61.05, 61.17), H (4.39, 4.44), N (5.09, 5.21). The formation of [Ru((E,E-4′-bipyridine)(E,E-4′-Bis[p-N,N-dimethylaniline])styril)-2′-bipyridine)]PF6 (2): (E,E)-4′-Bis[p-N,N-dimethylaniline]styril)-2′-bipyridine (338 mg, 0.76 mmol, 4.0 equiv.) and Ru(DMSO)2Cl2 (92 mg, 0.19 mmol, 1.0 equiv.) and lithium chloride (401 mg, 4.96 mmol, 50 equiv.) were dissolved in dry N,N-dimethylformamide (50 mL) under nitrogen atmosphere. The mixture was refluxed for 48 h. The solution was cooled down and a saturated aqueous solution of ammonium hexafluoroarsenate was added. The crude product, which precipitated as a hexafluoroarsenate salt was collected by centrifugation and washed with ethanol, water and diethyl ether. The residue was dissolved in dichloromethane and washed with 5% aqueous lithium chloride solution, brine and water. The solution was removed under reduced pressure and the crude product recrystallized from dichloromethane/pentane. The product was isolated via fractionated precipitation from acetonitrile (with a high ratio of acetonitrile and water). The solid was dried under vacuum and 86 mg of 2 (0.10 mmol, 31%) were yielded as black blocks. 1H-NMR (500 MHz, CDCl3): δ 8.82 (d, J = 1.9 Hz, 6H), 7.66 (d, J = 16.2 Hz, 6H), 7.65 (d, J = 6.1 Hz, 6H), 7.55–7.52 (m, 12H), 7.38 (dd, J = 6.1, 1.9 Hz, 6H), 7.03 (d, J = 16.2 Hz, 6H), 6.81–6.78 (m, 12H), 3.01 (s, 12H), 1.31 (s, 36H), 1.17 (d, J = 7.1 Hz, 6H), 0.89 (s, 3H), 0.80 (s, 3H). The mixture was then cooled down and undissolved solid was removed by filtration. A saturated aqueous solution of ammonium hexafluoroarsenate was added and the crude product, which precipitated as a hexafluoroarsenate salt was collected by filtration. The solids were washed with water and then dried at 50 °C. The residue was purified via fractionation from acetonitrile by adding dropwise diethyl ether. The collected product was dissolved in dichloromethane and washed with 5% aqueous lithium chloride solution, brine and water. After drying, 218 mg of 3 (0.13 mmol, 76%) were yielded as a black solid. 1H-NMR (500 MHz, CDCl3): δ 8.79 (d, J = 1.7 Hz, 6H), 7.77 (d, J = 16.4 Hz, 6H), 7.71 (d, J = 6.0 Hz, 6H), 7.64 (d, J = 8.9 Hz, 6H), 7.38 (s, 18H), 1.31 (s, 36H), 1.17 (d, J = 7.1 Hz, 6H), 0.89 (s, 3H). 13C-NMR (125 MHz, CDCl3): δ 152.0, 152.2, 148.0, 137.0, 130.1, 125.0, 122.7, 112.3, 115.5, 56.2; HRMS (m/z): [M]+2 calcd. for C78H60N6Ru, 591.1956; found, 589.67 (C84H72F12N6O6P2Ru): C (61.05, 61.17), H (4.39, 4.44), N (5.09, 5.21). [Ru(4′-bipyridine)(E,E-4′-Bis[p-N,N-dimethylaniline])styril)-2′-bipyridine)]Cl2 (4): [Ru(4′-bipyridine)(E,E-4′-Bis[p-N,N-dimethylaniline])styril)-2′-bipyridine)]Cl2 (200 mg, 0.49 mmol, 2.0 equiv.), Ru(DMSO)2Cl2 (119 mg, 0.25 mmol, 1.0 equiv.) and lithium chloride (1044 mg, 24.63 mmol, 100 equiv.) were suspended in dry N,N-dimethylformamide (30 mL) under nitrogen atmosphere. The mixture was refluxed for 15 h. The solution was then cooled down and undissolved solid was removed by filtration. A saturated aqueous solution of ammonium hexafluoroarsenate was added and the crude product, which precipitated as a hexafluoroarsenate salt was collected by filtration. The solids were washed with water and then dried at 50 °C. The residue was purified via fractionation from acetonitrile by adding dropwise diethyl ether. 449 mg of 6 (0.39 mmol, 54%) were yielded as a dark red solid. 1H-NMR (500 MHz, CDCl3): δ 8.52 (d, J = 1.7 Hz, 6H), 8.50 (d, J = 16.2 Hz, 2H), 7.87–8.04 (m, 2H), 7.59–7.74 (m, 2H), 7.52–7.49 (d, J = 6.0 Hz, 2H), 7.44–7.34 (m, 6H), 7.02 (d, J = 16.3 Hz, 2H), 6.81–6.76 (m, 4H), 3.01 (s, 12H). 13C-NMR (125 MHz, CDCl3): δ 158.0, 158.0, 152.7, 152.6, 151.9, 148.9, 138.6, 138.0, 130.0, 128.5, 128.2, 127.0, 121.1, 115.5, 56.2; HRMS (m/z): [M]+2 calcd. for C59H49NO10Ru, 549.1698; found, 549.1707; analysis (calcld., found for C44H36Fu6O10P2Ru + C18H18): H (4.44, 0.88), N (5.47, 5.79). [Ru(4′-bipyridine)(E,E-4′-Bis[p-N,N-dimethylaniline])styril)-2′-bipyridine)]PF6 (5): Ru(2′,2′-bipyridine)]Cl2 (350 mg, 0.72 mmol, 1.0 equiv.) and (E,E)-4′-Bis[p-N,N-dimethylaniline]styril)-2′-bipyridine) (388 mg, 0.87 mmol, 1.2 equiv.) were suspended in dry ethanol (50 mL) under nitrogen atmosphere and the mixture was refluxed for 6 h. Then the solution was cooled down and a saturated aqueous solution of ammonium hexafluoroarsenate was added. The crude product, which precipitated as a hexafluoroarsenate salt was collected by filtration and washed with water and diethyl ether. The product was isolated via fractionated precipitation from acetonitrile by adding dropwise diethyl ether. 449 mg of 6 (0.39 mmol, 54%) were yielded as a dark red solid. 1H-NMR (500 MHz, CDCl3): δ 8.52 (d, J = 1.7 Hz, 6H), 8.50 (d, J = 16.2 Hz, 2H), 7.87–8.04 (m, 2H), 7.59–7.77 (m, 2H), 7.52–7.49 (d, J = 6.0 Hz, 2H), 7.44–7.34 (m, 6H), 7.02 (d, J = 16.3 Hz, 2H), 6.81–6.76 (m, 4H), 3.01 (s, 12H). 13C-NMR (125 MHz, CDCl3): δ 158.0, 158.0, 152.7, 152.6, 151.9, 148.9, 138.6, 138.0, 130.0, 128.5, 128.2, 127.0, 121.1, 115.5, 56.2; HRMS (m/z): [M]+2 calcd. for C59H49NO10Ru, 549.1698; found, 549.1707; analysis (calcld., found for C44H36Fu6O10P2Ru + C18H18): H (4.44, 0.88), N (5.47, 5.79).
in acetonitrile ($\Phi_{\text{em}} = 5.9\%$) applying the following formula:

$$\Phi_{\text{em, sample}} = \frac{\Phi_{\text{em, reference}} \times (I_{\text{reference}}/I_{\text{sample}})}{(S_{\text{sample}}/S_{\text{reference}}) \times (F_{\text{sample}}/F_{\text{reference}})^2},$$

$$F = 1 - 10^{-\lambda}.$$  

Lifetime. The sample was prepared in an air saturated as well as degassed acetonitrile solution with an absorption of 0.2 at 355 nm. The sample was irradiated at 355 nm with a NT342B Nd:YAG pumped optical parametric oscillator (Ekspla). The emission was focused at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. The signal was detected with a R928 photomultiplier tube (Hamamatsu).

Electron spin resonance (ESR). The sample (10 µM) was dissolved in acetonitrile or PBS containing 20 mM TEMP (2,2,6,6-tetramethylpiperidine) as a 1$\alpha$O$_2$ scavenger or 20 mM DMPO (5,5-dimethyl-1-pyrrolidine N-oxide) as a "OOH or $^\bullet$OH radical scavenger. Capillary tubes were filled with the solution and sintered by fire. EPR spectra were recorded on a Bruker A380 spectrometer with 1 G field modulation, 100 G scan range, and 20 mW microwave power. The samples were measured in exclusion from light and after irradiation (450 ± 10 nm, 60 s, and 21.8 mW cm$^{-2}$).

Singlet oxygen. Direct evaluation: The sample was prepared in an air-saturated acetonitrile or deuterated water solution with an absorption of 0.2 at 450 nm. The sample was irradiated at 450 nm with a mounted M450LP1 LED (Thorlabs), whose light was focused with aspheric condenser lenses. Using a T-Cube LED Driver (Thorlabs), the intensity of the irradiation was varied and monitored with an optical power and energy meter. The emission was focused at right angle to the excitation pathway and directed to a Princeton Instruments Acton SP-2300i monochromator. To cut off light at wavelengths shorter than 850 nm, a longpass glass filter was placed in front of the monochromator entrance slit. The signal was detected with an EO-817L IR-sensitive liquid-nitrogen cooled mercury cadmium telluride detector (North Coast Scientific Corp.). The luminescence signal, centered at 1270 nm, was measured from 1100 to 1400 nm. The obtained data were analyzed upon plotting the integrated luminescence peaks against the percentage of the irradiation intensity. The slope of the linear regression was calculated and compared with the reference Rose Bengal ($\Phi = 76\%$)47. The absorption of the sample was corrected with an absorption correction factor. The singlet oxygen quantum yields were calculated using the following formula:

$$\Phi_{\text{sample}} = \Phi_{\text{reference}} \times \frac{S_{\text{sample}}/S_{\text{reference}}}{I_{\text{reference}}/I_{\text{sample}}}.$$

$$I = I_{0} \times (1 - 10^{-\lambda}).$$

$$\Phi = \text{singlet oxygen quantum yield}, S = \text{slope of the linear regression of the plot of the areas of the singlet oxygen luminescence peaks against the irradiation intensity}, I_0 = \text{light intensity of the irradiation source}, A = \text{absorption of the sample at irradiation wavelength}.$$  

Indirect evaluation: measurement in acetone: the sample was prepared in an air-saturated acetonitrile solution with an absorption of 0.2 at the irradiation wavelength, N,N-dimethyl-4-nitrosoaniline aniline (RNO, 20 µM) and histidine (10 mM). The samples were irradiated for various time points with an Atlas Photonics LUMOS BIO irradiator. The absorption of the samples was constantly measured in exclusion from light and after irradiation (440 ± 10 nm, 60 s, and 21.8 mW cm$^{-2}$).

Stability in human plasma. The stability of the sample was evaluated upon incubation in pooled human plasma with caffeine as an internal standard, which previously demonstrated to be stable under these conditions$^{46}$. The sample (20 µM) and caffeine (40 µM) were prepared in dimethyl sulfoxide stock solutions. One aliquot of both solutions was added to 975 µL of human plasma achieving a total volume of 1000 µL. The resulting solutions were incubated upon continuous gentle shaking (ca. 300 rpm) for 48 h at 37°C. After this time, the incubation was ended by addition of 3 mL of methanol. The mixture was centrifuged for 60 min at 3000 rpm/958 g and 4°C. The resulting supernatant was filtered through a 0.2 µm membrane filter. The solvent was evaporated and the residue was redissolved in 1:1 (v/v)
acetonitrile/water with 0.1% trifluoroacetic acid. The resulting solution was then filtered through a 0.2 μm membrane filter and analyzed with a HPLC System. The solvent was isocratic 95% acetonitrile/5% water (solvent A) and acetonitrile (solvent B). Method M1: 0–3 min: isocratic 50% A (50% B); 3–17 min: linear gradient from 50% A (50% B) to 0% A (100% B); Method M2: 0–3 min: isocratic 95% A (5% B); 3–17 min: linear gradient from 95% A (5% B) to 0% A (100% B); 17–23 min: isocratic 0% A (100% B). The flow rate was 1 mL min⁻¹ and the chromatogram was detected at 230 nm.

**Photostability.** The sample was prepared in an air-saturated acetonitrile solution. Using a Atlas Photonics LUMOS BIO irradiator, the sample was constantly irradiated at 450 nm in time intervals from 0 to 10 min (13.2 J cm⁻²). The absorption spectra from 350 to 700 nm was monitored with a SpectrAmax M2 Microplate Reader (Molecular Devices) at each time interval. As positive and negative controls, respectively, [Ru(2,2'-bipyridine)]Cl₂ and Protoporphyrin IX were used.

**Photobleaching.** The sample was dissolved in air saturated 0.7 mL CD₃CN and stored in a NMR tube in the dark. A ¹H-NMR spectrum was measured directly after preparation to ensure the identity of the sample. The sample was then irradiated at 450 nm with an Atlas Photonics LUMOS BIO irradiator (10 min, 13.2 J cm⁻²). Following this treatment, a ¹H-NMR spectrum was again measured.

**Distribution coefficient.** Using the “shake-flask” method, the distribution coefficient between the PBS and octanol phase of the sample was determined. The PBS and octanol phases were previously saturated in each other. The sample was dissolved in each phase which is dissolving the best the compound. This solution was added to an equal volume of the other phase and mixed at 190 rpm overnight with a sample mixer. Following this, the solutions were equilibrated for 4 h. The phases were carefully separated from each other. The amount of the sample in each phase was determined by ICP-MS. The evaluation was repeated three times and the ratio between the organic and aqueous phase determined.

**Cell culture.** Human glioblastoma astrocytoma (U373) cells were cultured in MEM medium supplemented with 10% FBS, 1% NEAA (nonessential amino acids), and 1% penicillin/streptomycin. Human cervical carcinoma (HeLa), doxorubicin-resistant human colon adenocarcinoma (SW620/AD300), and the mouse colon carcinoma (CT-26) cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. Human retinal epithelial (RPE-1) cells were cultured in DMEM-F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were obtained from the American Type Culture Collection and cultured at 37 °C and 5% CO₂. Before an experiment, the cells were passaged three times.

**Time-dependent cellular uptake.** A total of 6 × 10⁵ cells were incubated with the sample (10 μM, 2% DMSO, v/v) for varying time points (0.5, 1, 2, 4, 8, 12 h) at 37 °C. After this time, the media was removed and the cells washed with cell media. The culture supernatant was harvested, centrifuged and resuspended. The number of cells on the dish was counted. The sample was diluted using a 60% HNO₃ solution for 3 days and following this, diluted to a 2% HNO₃ solution in water. The Ru content was determined with an ICP-MS apparatus and the results compared with the Ru references. The Ru content was then associated with the number of cells.

**Cellular uptake mechanism.** The cellular uptake mechanism was investigated upon pretreatment of 1 × 10⁶ cells with pathways inhibitors and determination of the amount of Ru inside the cells with an ICP-MS apparatus.

**Control:** the cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 1 h at 37 °C and then washed with PBS. The cells were detached with trypsin, harvested, centrifuged, and resuspended. The number of cells on the dish was counted. The sample was diluted using a 60% HNO₃ solution for 3 days as well as following this, diluted to a 2% HNO₃ solution in water. The Ru content was determined with an ICP-MS apparatus and the results compared with the Ru references. The Ru content was then associated with the number of cells.

**Low temperature metabolic inhibition:** the cells were preincubated with ammonium chloride (50 mM) or chloroquine (100 μM) for 1 h. After this time, the cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 1 h at 37 °C and then washed with PBS. The cells were detached with trypsin, harvested, centrifuged, and resuspended. The number of cells on the dish was counted. The sample was digested using a 60% HNO₃ solution for 3 days and following this diluted to a 2% HNO₃ solution in water. The Ru content was determined with an ICP-MS apparatus and the results compared with the Ru references. The Ru content was then associated with the number of cells.

**Two-photon induced cellular singlet oxygen generation.** A total of 1 × 10⁶ cells were seeded on confocal dishes and allowed to adhere overnight. The cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 4 h at 37 °C in the dark. After this time, the cells were washed with PBS. The cells were further incubated with LysoTracker Green, MitoTracker Deep Red, Cell-light Golgi-GFP, ER tracker Green and Hoechst 33342 for 30 min at 37 °C in the dark. The cells were washed three times with PBS. Confocal images were taken with a LSM 880 (Carl Zeiss) laser scanning confocal microscope. The Tdtلل trackers were excited and detected as recommended by the supplier. The samples were detected under usage of their 1-Photon (λex = 458 nm, λem = 600–750 nm) or 2-Photon (λex = 800 nm, λem = 600–750 nm) luminescence properties.

**Intracellular distribution by ICP-MS.** A total of 10 × 10⁶ cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 8 h at 37 °C in the dark. After this time, the cells were washed with PBS. The cells were further incubated with LysoTracker Green, MitoTracker Deep Red, Cell-light Golgi-GFP, ER tracker Green and Hoechst 33342 for 30 min at 37 °C in the dark. The cells were washed three times with PBS. Confocal images were taken with a LSM 880 (Carl Zeiss) laser scanning confocal microscope. The Lyso tracker Green was excited and detected as recommended by the supplier. The samples were detected under usage of their 1-Photon (λex = 458 nm, λem = 600–750 nm) or 2-Photon (λex = 800 nm, λem = 600–750 nm) luminescence properties.

**Intracellular distribution by confocal luminescence imaging.** A total of 1 × 10⁶ cells were seeded on confocal dishes and allowed to adhere overnight. The cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 1 h at 37 °C in the dark. After this time, the cells were incubated with 2′-dichlorofluorescein diacetate for 30 min. The cells were washed three times with PBS. The sample was diluted using a 60% HNO₃ solution for 3 days and following this diluted to a 2% HNO₃ solution in water. The Ru content was determined with an ICP-MS apparatus and the results compared with the Ru references. The Ru content was then associated with the number of cells.

**One-photon induced cellular singlet oxygen generation.** A total of 1 × 10⁶ cells were seeded on confocal dishes and allowed to adhere overnight. The cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 4 h at 37 °C in the dark. After this time, the cells were washed with PBS. The cells were further incubated with 2′-dichlorofluorescein diacetate for 30 min. The cells were washed three times with PBS. The sample was irradiated upon 2-Photon excitation (λem = 800 nm). Confocal images (λex = 488 nm, λem = 510–550 nm) were taken with a LSM 880 (Carl Zeiss) laser scanning confocal microscope.

**Two-photon induced cellular singlet oxygen generation.** A total of 1 × 10⁶ cells were seeded on confocal dishes and allowed to adhere overnight. The cells were incubated with the sample (10 μM, 2% DMSO, v/v) for 4 h at 37 °C in the dark. After this time, the cells were washed with PBS. The cells were further incubated with 2′-dichlorofluorescein diacetate for 30 min. The cells were washed three times with PBS. The sample was irradiated upon 2-Photon excitation (λem = 800 nm). Confocal images (λex = 488 nm, λem = 510–550 nm) were taken with a LSM 880 (Carl Zeiss) laser scanning confocal microscope.

**Photo-cytotoxicity on 2D cell monolayers.** A total of 4 × 10⁵ cells were seeded on 96-well plates and allowed to adhere overnight. The cells were treated with increasing concentrations of the sample diluted in cell media achieving a total amount of Ru inside the cells with an ICP-MS apparatus.
0.2 mg mL\(^{-1}\). The cells were incubated for 4 h and the amount of the fluorescent product resorufin determined (\(\lambda_{\text{ex}} = 540\ nm\), \(\lambda_{\text{em}} = 590\ nm\)) with a SpectraMax M2 Microplate Reader (Molecular Devices). The obtained data was analyzed with the GraphPad Prism software.

**Cell death mechanism.** The cell death mechanism was investigated by measuring the cell viability upon preincubation with various cell death pathway inhibitors. 3-Methyladenine (100 \(\mu\)M), Z-VAD-FMK (20 \(\mu\)M), cycloheximide (0.1 \(\mu\)M), and necrostatin-1 (60 \(\mu\)M) were preincubated with the cells for 40 min. Following this, the sample (5 \(\mu\)M) was added and incubated for 8 h. The cells were washed, irradiated with a LED (500 nm, 10 min, 10.0 mW cm\(^{-2}\), 6 \(\mu\)m\(^{-2}\)) light source, and incubated for 12 h. After this time, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added and the cells were incubated for additional 4 h. The liquid was disposed and 150 \(\mu\)L DMSO was added. The plates were shaken for 2 min and the absorbance at 595 nm was measured using a microplate reader (TecanInfiniteF200, Bio-rad).

**Generation and analysis of 3D multicellular tumor spheroids (MCTS).** A suspension of 0.75% agarose in PBS was heated in a high-pressure autoclave. The hot emulsion was transferred into a 96-well plate (50 \(\mu\)L per well). The plates were exposed for 3 h to UV irradiation and allowed to cool down. After this time, a cell suspension of 3 \(\times\) 10\(^4\) cells was seeded on top the agarose layer. Within 2–3 days, MCTS were formed from the cell suspension. The MCTS were cultured and maintained at 37 °C in a cell culture incubator at 37 °C with 5% CO\(_2\) atmosphere. The cell culture medium was replaced every 2 days. The formation, integrity, diameter, and volume of the MCTS were monitored with an Axio Observer Z1 (Carl Zeiss) phase contrast microscope. The volume was calculated using the following equation:

\[
V = \frac{4}{3} \pi r^3.
\]

The luminescence images along the z-axis were captured by a LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with a Coherent Chameleon 2-Photon laser. The cell culture media was replaced every 2 days. The integrity and diameter of the MCTS was monitored with an Axio Observer Z1 (Carl Zeiss) phase contrast microscope every 24 h.

**3D multicellular tumor spheroids (MCTS) growth inhibition assay.** MCTS were treated with the sample (20 \(\mu\)m, 2% DMSO, \%) by replacing 50% of the media with drug supplemented media in the dark for 3 days. After this time, the MCTS were exposed to a 1-Photon (500 nm, 16.7 min, 10.0 mW cm\(^{-2}\), and 10 \(\mu\)m\(^{-2}\)) irradiation using a LED light source or 2-Photon (800 nm, 10 \(\mu\)m\(^{-2}\)) with a section interval of 5 \(\mu\)m irradiation using a LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with a Colher Chameleon 2-Photon laser. The cell culture media was replaced every 2 days. The integrity and diameter of the MCTS was monitored with an Axio Observer Z1 (Carl Zeiss) phase contrast microscope every 24 h.

**3D multicellular tumor spheroids (MCTS) viability assay.** MCTS were treated with the sample (20 \(\mu\)m, 2% DMSO, \%) by replacing 50% of the media with drug supplemented media in the dark for 3 days. After this time, the MCTS were exposed to a 1-Photon (500 nm, 16.7 min, 10.0 mW cm\(^{-2}\), and 10 \(\mu\)m\(^{-2}\)) irradiation using a LED light source or 2-Photon (800 nm, 10 \(\mu\)m\(^{-2}\)) with a section interval of 5 \(\mu\)m irradiation using a LSM 880 (Carl Zeiss) laser scanning confocal microscope equipped with a Coherent Chameleon 2-Photon laser. The cell culture media was replaced every 2 days. The volume was calculated using the following formula:

\[
V = \frac{4}{3} \pi r^3.
\]

**Histological examination.** The tumor and all major organs (including the heart, liver, spleen, lung, kidney, brain, intestine, and blood) were collected and a slice of each one was fixated with 4% paraformaldehyde. The obtained slices were stained with hematoxylin and eosin. The histological images were taken with a Carl Zeiss Axio Imager Z2 microscope.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the paper and its Supplementary Information files. Correspondence and requests for materials should be addressed to H.C. or G.G.

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Author contributions
All authors were involved with the design and interpretation of experiments and with the writing of the paper. DFT calculations were performed by J.K. and F.M. Chemical, photophysical, and biological experiments were carried out by J.K. X-ray crystallography was carried out by J.K. and O.B. Animal testing was carried out by J.K. and S.K. The work was supervised by I.C., H.C., and G.G. All authors have given approval to the final version of the paper.

Competing interests
The authors declare no competing interests.

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