

Supporting Information

Engineered flavoproteins as bioorthogonal photo-triggers for the activation of metal-based anticancer prodrugs

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1. General methods	S2
2. Synthesis and characterization	S4
3. Protein design and characterization	S14
4. Bioconjugation of CTPRs	S15
5. Photostability	S18
6. Photocatalysis studies	S19
7. Incubation experiments with GSH	S21
8. Cell viability experiments	S22
9. References	S23

1. General methods

Materials. Chemicals, solvents and deuterated solvents for NMR were obtained from Merck and used as purchased without further purification. Ultrapure water was deionised and filtered with a Millipore milliQ system.

Column chromatography was performed with silica gel employing Macherey-Nagel Kieselgel 60 with particles sizes of 0.063–0.2 mm (gravity).

NMR spectroscopy. NMR spectra for characterization work were acquired on a Bruker 500 MHz Ultra Shield and Bruker Fourier 300 MHz spectrometer. Spectra were calibrated using residual solvent signals according to previously reported values.¹

UV-Vis spectroscopy. UV-Vis spectra were acquired with Biotek Synergy H1 microplate reader, NanoDrop Spectrophotometer ND-1000 and NanoDrop One^c Spectrophotometer. For the microplate reader measurements, 96 well UV flat bottom MicrotiterPlates (Thermo Fisher) were used. For both NanoDrop instruments, the measurements were performed loading 3.5 μ l of sample.

Circular dichroism (CD). CD was used to determine the secondary structure of the CTPR units. CD was performed using a Jasco J-1500 spectropolarimeter. A quartz cuvette (0.1 mm path length) was used. The CD spectra were acquired at 1 nm increments and 2 seconds average time over a wavelength range of 190 to 260 nm.

Mass spectrometry.

ESI-MS. ESI (Electrospray ionization) mass spectra were recorded on an Agilent Technologies 1100 Series system equipped with a binary pump (G1312A) and MSD SL Trap mass spectrometer (G2445D SL) with ESI source from solutions in methanol or acetonitrile and 0.1% formic acid.

MALDI-TOF MS. The proteins and the organic compounds were analyzed using MALDI-TOF MS UltrafleXtreme III (Bruker) mass spectrometer. 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid was used as matrix at 10 mg/ml dissolved in a 70% acetonitrile and 0.1% TFA solution for proteins samples, 2,5-dihydroxybenzoic acid was used as matrix at 20 mg/ml dissolved in 70% acetonitrile and 0.1% TFA for organic compound. The samples were prepared at a sample to matrix solution ratio of 1:4 (v/v) and 5 μ l of the mixture deposited on the sample plate.

ICP-MS. The amount of platinum in conjugated samples and internalized in PANC-1 cancer cell lines, was determined by ICP-MS iCAP-Q (ThermoFisher) spectrometer. The samples were digested with aqua regia (overnight) and diluted with water before the measurements.

LC-MS. LC (Liquid chromatography) mass spectrometry was carried out using a UHPLC chromatography system (Acquity UPLC, Waters, Milford, USA) coupled with an ESI-TOF mass spectrometer (LCT Premier XE, Waters, Milford, USA). The chromatographic separation was performed on an Acquity BEH reversed phase C18 column, using a mobile phase consisting of 100 mM ammonium formate in water (A) and acetonitrile (B). The gradient method was as follows: 0-1.5 min at 99% A, 1.5-6 min to 1% A, 6-8 min at 1% A, 8-8.5 min to 99% A, 8.5-10 min to 99%. The mass spectrometry detection was accomplished working in positive / W mode. The MS range acquired was between m/z 100-1000. The capillary and cone voltages were set at 1500 and 100V, respectively. Masslynx v4.1 software was used to analyze chromatograms and spectra (Waters, Milford, MA, USA). The use of ammonium formate as a buffer in the aqueous mobile phase enables the detection of platinum complexes as ammonium adducts. Similarly, the presence of platinum can be confirmed by comparing experimental isotopic distributions with calculated ones.

Gel electrophoresis. 15% SDS-PAGE electrophoresis gels were used to monitor the conjugation reaction. Low range protein was used as marker (LMW II) and the conjugated fractions were mixed with SDS loading buffer. The gel was first imaged using UV-light to monitor the fluorescence of the functionalized riboflavin and then stained with Coomassie Blue.

Cell culture experiments. PANC-1 pancreatic cancer cell line was obtained from the American Type Tissue Collection (ATCC) and cultured in DMEM/F-12 medium (without phenol red) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO₂. Cells were passed at 70% of confluence.

Cell viability was measured using resazurin assay. For the measurements, PANC-1 were seeded in 96-well plates with a density of 7500 cells per well and allowed to adhere overnight. The medium was then substituted with fresh complete medium containing the samples at the desired concentration and cells were incubated for another 24 hours. The medium was replaced by a solution mixture of DMEM/F-12 medium and resazurin, after a wash with 200 µl of DPBS, and the cells incubated at 37 °C for 3 hours. Viability measurements were then conducted at 37 °C. The samples were excited at 560 nm and the fluorescence collected at 590 nm. Fluorescence spectra were recorded with Biotek Synergy H1 microplate reader using sterilized Nuclon Delta Surface 96 well (Thermo Fisher Scientific). Cell viability was expressed as percent value calculated as:

$$\text{Cell viability (\%)} = 100 - (\text{F.I.}_T / \text{F.I.}_C * 100)$$

Where F.I._T is the fluorescence of treated cells and F.I._C is the fluorescence of the untreated cells.

For the cell irradiation experiments, blue light (460 nm, 3.5 mW/cm²) was used. Cells were seeded, incubated for 24 hours with samples and washed with 200 µl of DPBS before being irradiated for 45 min. It was alternated a time period of 10 min light on/off, 15 min for the last irradiation section. Cell viability was then measured with resazurin assay, as described before.

2. Synthesis and characterization

Synthesis of *N*-(3-(7,8-dimethyl-2,4-dioxo-10-((2*R*,3*R*,4*S*)-2,3,4,5-tetrahydroxypentyl)-4,10-dihydrobenzo[*g*]pteridin-3(2*H*)-yl)propyl)-2-iodoacetamide (Rf-IA)

The iodoacetamide-functionalized riboflavin was prepared following the procedure described in Fig. S1. Previously reported methods were followed for the preparation of compounds I², II³, and III.⁴

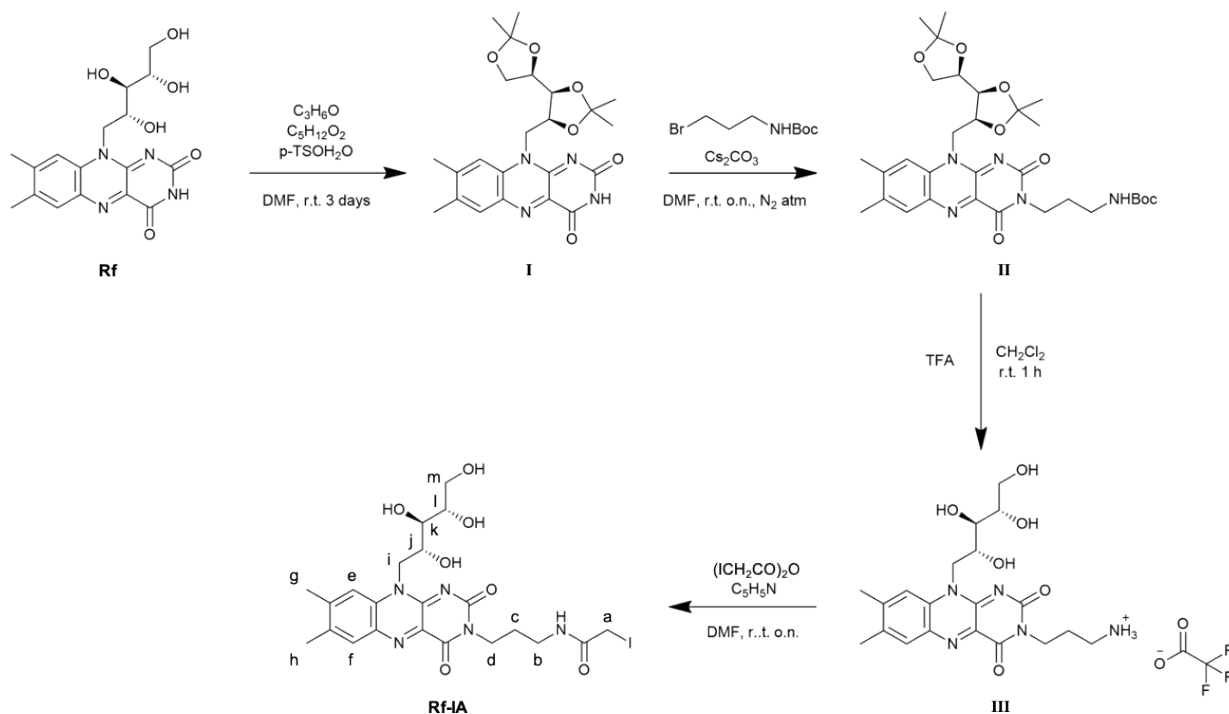
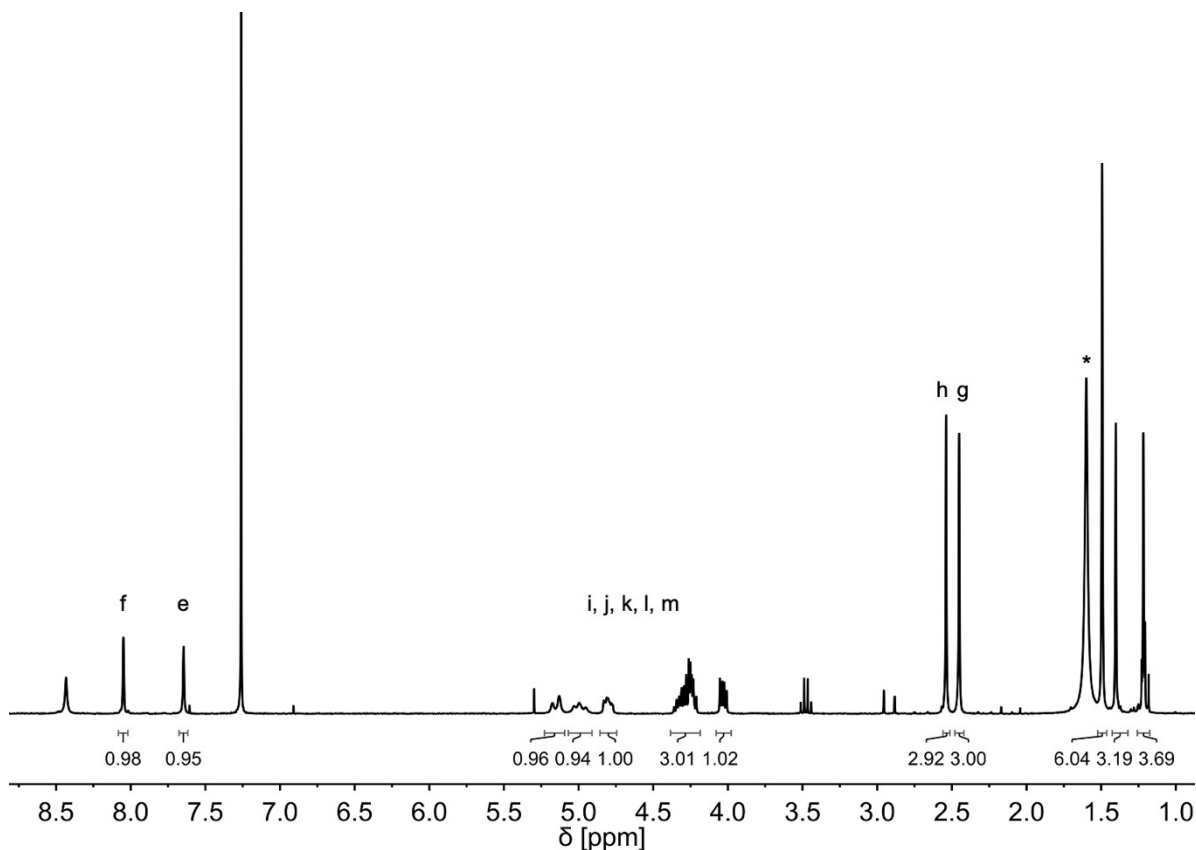


Fig. S1 Synthetic procedure adopted for the preparation of Rf-IA.

Step 1. 7,8-dimethyl-10-(((4*S*,4'*R*)-2,2,2',2'-tetramethyl-[4,4'-bi(1,3-dioxolan)]-5-yl)methyl)benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (I)

The starting point of our procedure was the protection of the hydroxyl groups of the ribityl chain by dimethyl acetal groups.² Commercial riboflavin (3 g) was dissolved in acetone (32 mL), dimethylformamide (40 mL), dimethoxypropane (16 mL) and p-toluenesulfonic acid monohydrate (1.5 g) was added. The resulting mixture was stirred at room temperature for 3 days. 80 mL of water were then added and the aqueous phase was extracted with CH₂Cl₂. The combined organic phases were dried over MgSO₄ and concentrated in vacuum. The product was then purified by centrifugation at 3500 rpm to remove DMF (after adding 3 mL of diethyl ether to the product) (2.5 g, 70%).

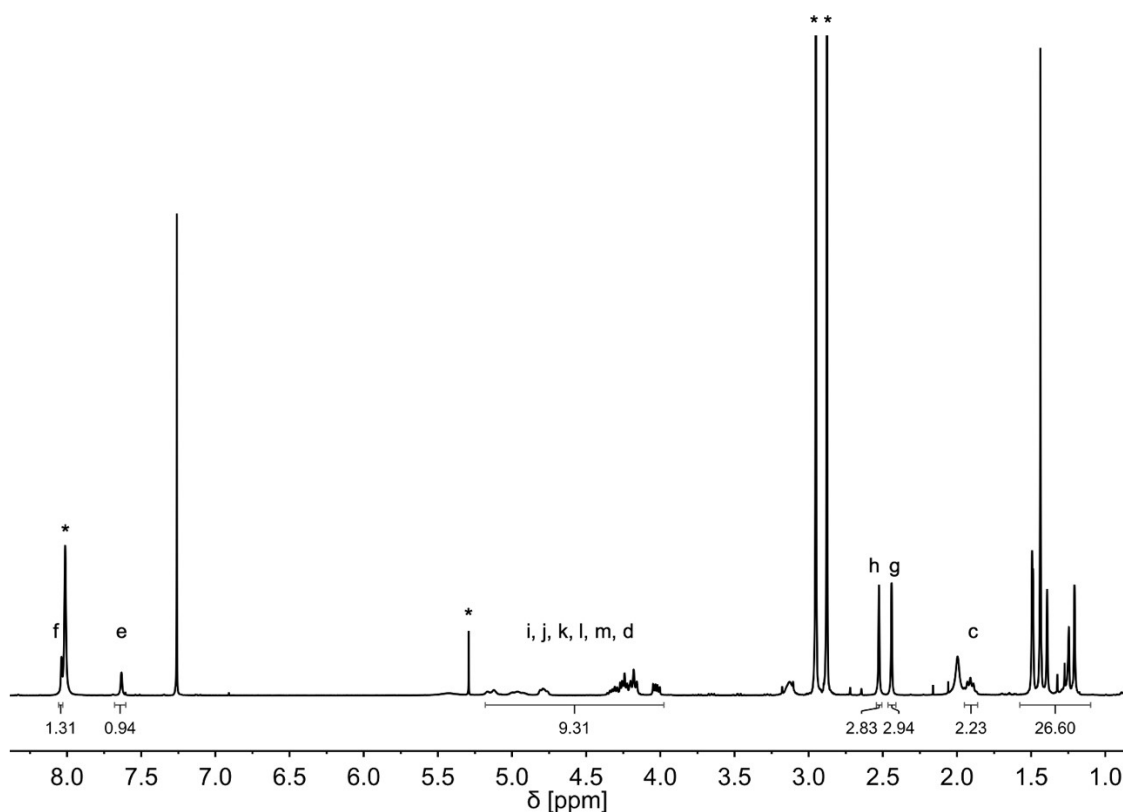
¹H-NMR (300 MHz, CDCl₃), δ(ppm): 8.05 (s, 1H, H_(f)), 7.65 (s, 1H, H_(e)), 5.23-3.97 (m, 7H, H_(i), H_(j), H_(k), H_(l), H_(m)), 2.53 (s, 3H, H_(h)), 2.46 (s, 3H, H_(g)), 1.52-1.20 (m, 12H, C(CH₃)₂) (* solvent impurities: 1.60 water).



Step 2. Tert-butyl (3-(7,8-dimethyl-2,4-dioxo-10-(((4S,4'R,5S)-2,2,2',2'-tetramethyl-[4,4'-bi(1,3-dioxolan)]-5-yl)methyl)-4,10-dihydrobenzo[g]pteridin-3(2H)-yl)propyl)carbamate (II)

Subsequently, a nucleophilic substitution to the isoalloxazine N3 was performed.³ The protected riboflavin (I) (0.470 g) and Cs₂CO₃ (0.505 g) were suspended in dry dimethylformamide (5 mL) and stirred under nitrogen for 30 min. Next, 3-(Boc-amino)propyl bromide was dissolved in 3 mL of dry DMF and added to the reaction mixture, which was left stirring overnight at room temperature under nitrogen. Afterwards, the mixture was portioned in CH₂Cl₂ and brine (saturated solution of NaCl) and the organic phase was washed with brine. The organic phase was kept, washed with MgSO₄, filtered and removed under vacuum. The brown oil obtained was purified by column chromatography (eluent CH₂Cl₂:MeOH 99:1) (0.47 g, 85%).

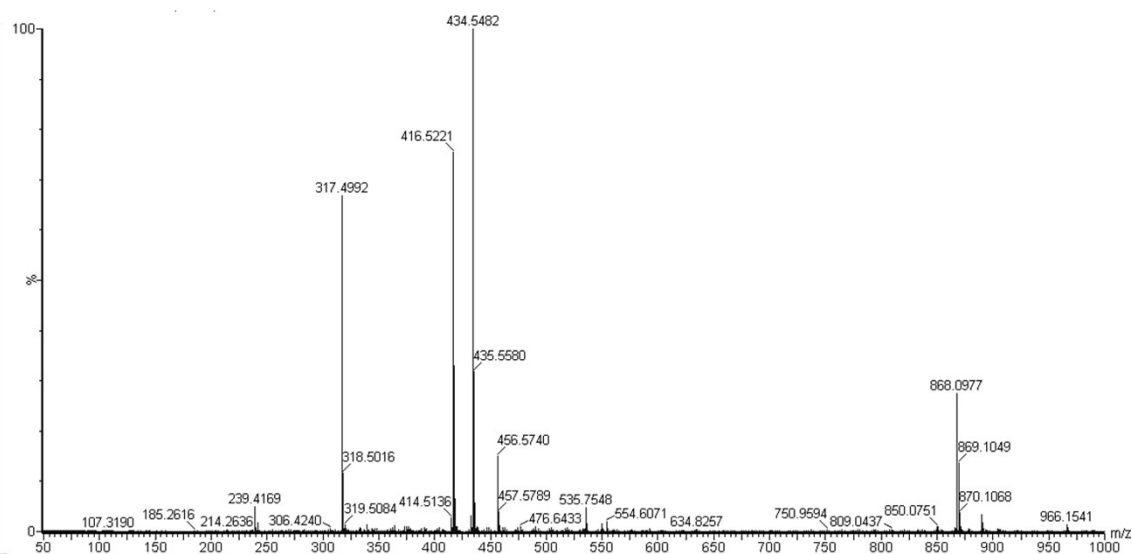
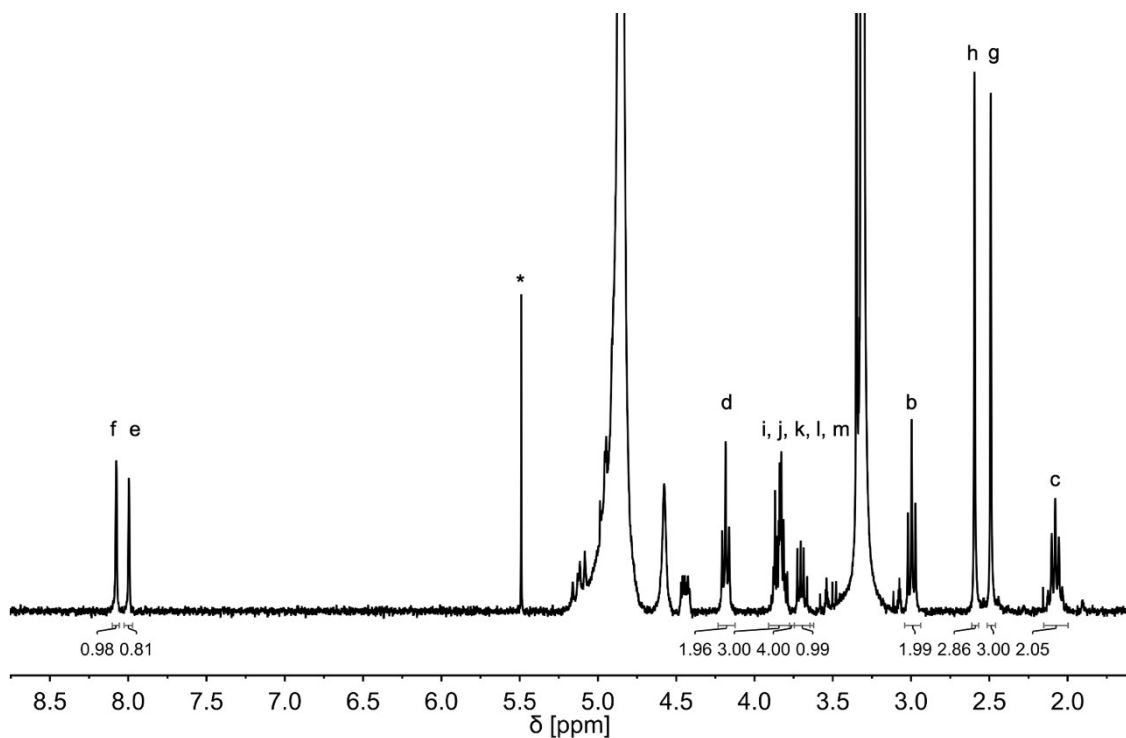
¹H-NMR (300 MHz, CDCl₃), δ(ppm): 8.01 (s, 1H, H_(f)), 7.62 (s, 1H, H_(e)), 5.20-3.98 (m, 9H, H_(i), H_(j), H_(k), H_(l), H_(m), H_(d)), 2.53 (s, 3H, H_(h)), 2.45 (s, 3H, H_(g)), 1.91 (p, 2H, J = 6.1 Hz, H_(c)), 1.52-1.17 (m, 21H, C(CH₃)₃). The H_(b) signal is covered by DMF peaks (* solvents impurities: 8.01, 2.95 and 2.87 dimethylformamide, 5.30 dichloromethane)).



Step 3. 3-(7,8-dimethyl-2,4-dioxo-10-((2R,3R,4S)-2,3,4,5-tetrahydroxy-pentyl)-4,10-dihydrobenzo[g]pteridin-3(2H)-yl)propan-1-aminium (III)

Protecting groups were removed adapting a previously reported procedure.⁴ The protected and functionalized riboflavin (0.160 g) was dissolved in 80 mL of CH₂Cl₂ in an ice bath under stirring. 3 mL of TFA were then added to the mixture and the ice bath was removed. The reaction was left stirring for 1 hour at room temperature. Then, the solvent was removed under vacuum and the product was washed four times with CH₂Cl₂. The final product was purified by column chromatography (eluent MeOH:H₂O 37:63) (0.066 g, 59 %).

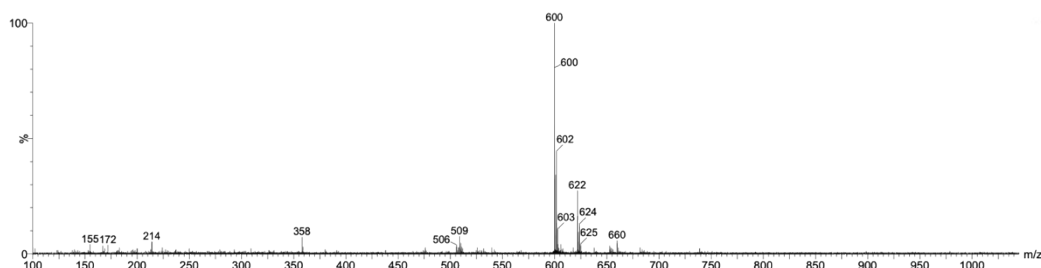
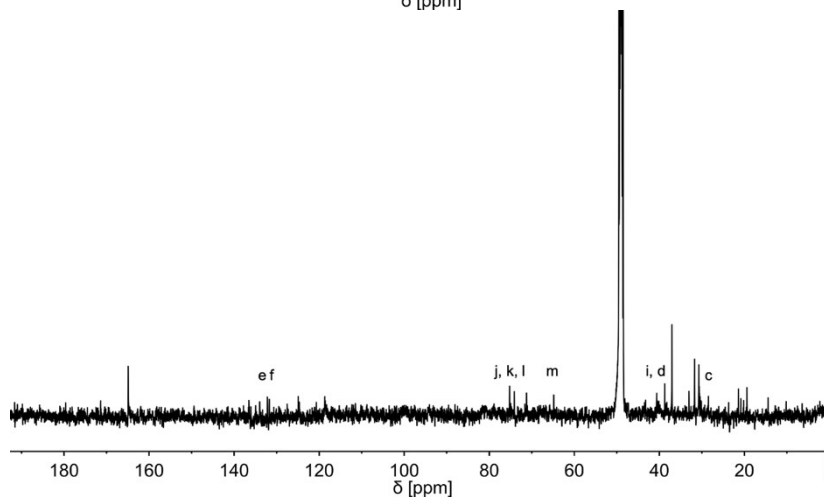
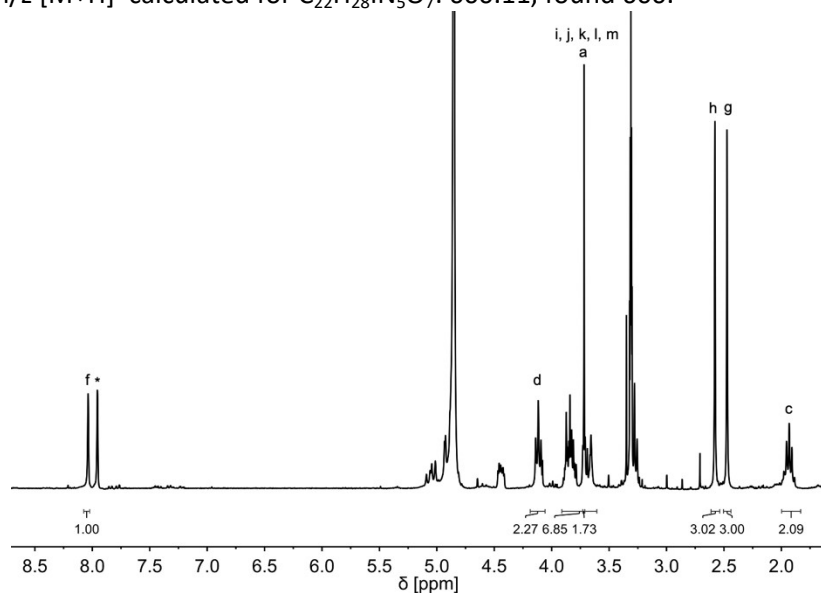
¹H-NMR (300 MHz, CD₃OD), δ(ppm): 8.02 (s, 1H, H_(f)), 7.96 (s, 1H, H_(e)), 4.18 (t, 2H, J = 6.6 Hz, H_(d)), 3.91-3.64 (m, 5H, H_(i), H_(j), H_(k), H_(l), H_(m)), 3.00 (t, 1H, J = 7.2 Hz, H_(b)), 2.6 (s, 3H, H_(h)), 2.49 (s, 3H, H_(g)), 2.08 (p, 2H, J = 6.9 Hz, H_(c)) (* solvent impurities: 5.49 dichloromethane). ESI-MS, m/z [M+H]⁺ calculated for C₂₁H₂₉N₄O₆: 433.21, found 434.5.



Step 4. *N*-(3-(7,8-dimethyl-2,4-dioxo-10-((2*R*,3*R*,4*S*)-2,3,4,5-tetrahydroxypentyl)-4,10-dihydrobenzo[*g*]pteridin-3(2*H*)-yl)propyl)-2-iodoacetamide (Rf-IA)

Functionalization of riboflavin with a terminal iodoacetamido group was achieved by reacting **III** with iodoacetic anhydride.⁵ Iodoacetic anhydride (26.00 mg) dissolved in 5 mL of dry DMF was added dropwise to a solution (5 mL, dry DMF) of the riboflavin derivative **III** (45.10 mg). This reaction step was performed in an ice bath, which we removed at the end of the addition. Afterwards, the reaction was left stirring overnight. The solvent was removed under vacuum and **Rf-IA** was subsequently purified by column chromatography (eluent MeOH:CH₂Cl₂ from 1:5 to MeOH:CH₂Cl₂ 1:3) (6.4 mg, 15%).

¹H-NMR (300 MHz, CD₃OD), δ(ppm): 8.06 (s, 1H, H_(f)), 4.13 (t, 2H, J = 6.9 Hz, H_(d)), 3.91-3.61 (m, 7H, H_(i), H_(j), H_(k), H_(l), H_(m)), 3.71 (s, 2H, H_(a)), 2.58 (s, 3H, H_(h)), 2.47 (s, 3H, H_(g)), 1.93 (p, 2H, J = 6.9 Hz, H_(c)). The H_(e), H_(b) signals are covered by DMF peaks (* solvent impurities: 7.95 dimethylformamide). ¹³C {¹H} (125.7 MHz, CD₃OD), δ(ppm): 132.13 (1C, C_(e)), 131.66 (1C, C_(f)), 127.49 (1C, CCN), 124.86 (1C, NCCN), 75.22-71.20 (3C, C_(j), C_(k), C_(l)), 64.79 (1C, C_(m)), 40.60-38.75 (2C, C_(i), C_(d)), 28.48 (1C, C_(c)), 21.40-19.40 (2C, C(CH₃)C, C(CH₃)C), -2.70 (1C, C_(a)). ESI-MS, m/z [M+H]⁺ calculated for C₂₂H₂₈N₅O₇: 600.11, found 600.



Synthesis of *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(OCOCH₃)(O₂CCH₂CH₂CO₂H)] (Pt1) and its NHS-activated derivative (Pt1-NHS)

The Pt1 complex was prepared following the procedure described in Fig. S2. Previously reported methods were adopted for the preparation of cisplatin^{6,7}, IV⁸, Pt1⁸ and Pt1-NHS.⁸

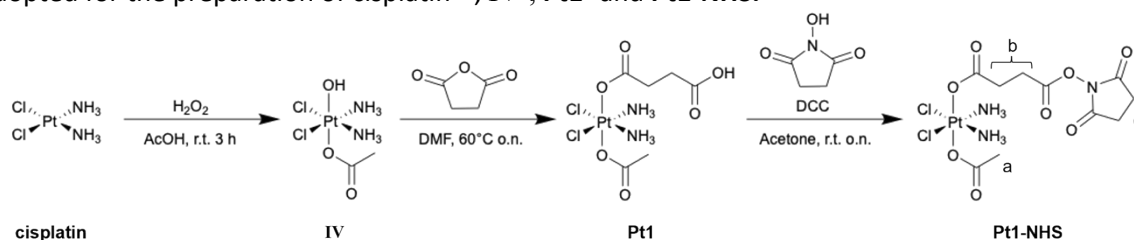


Fig. S2 Synthetic procedure for the preparation of Pt1 and Pt1-NHS.

Step 1. *cis*-diamminedichloroplatinum(II) (*cisplatin*)

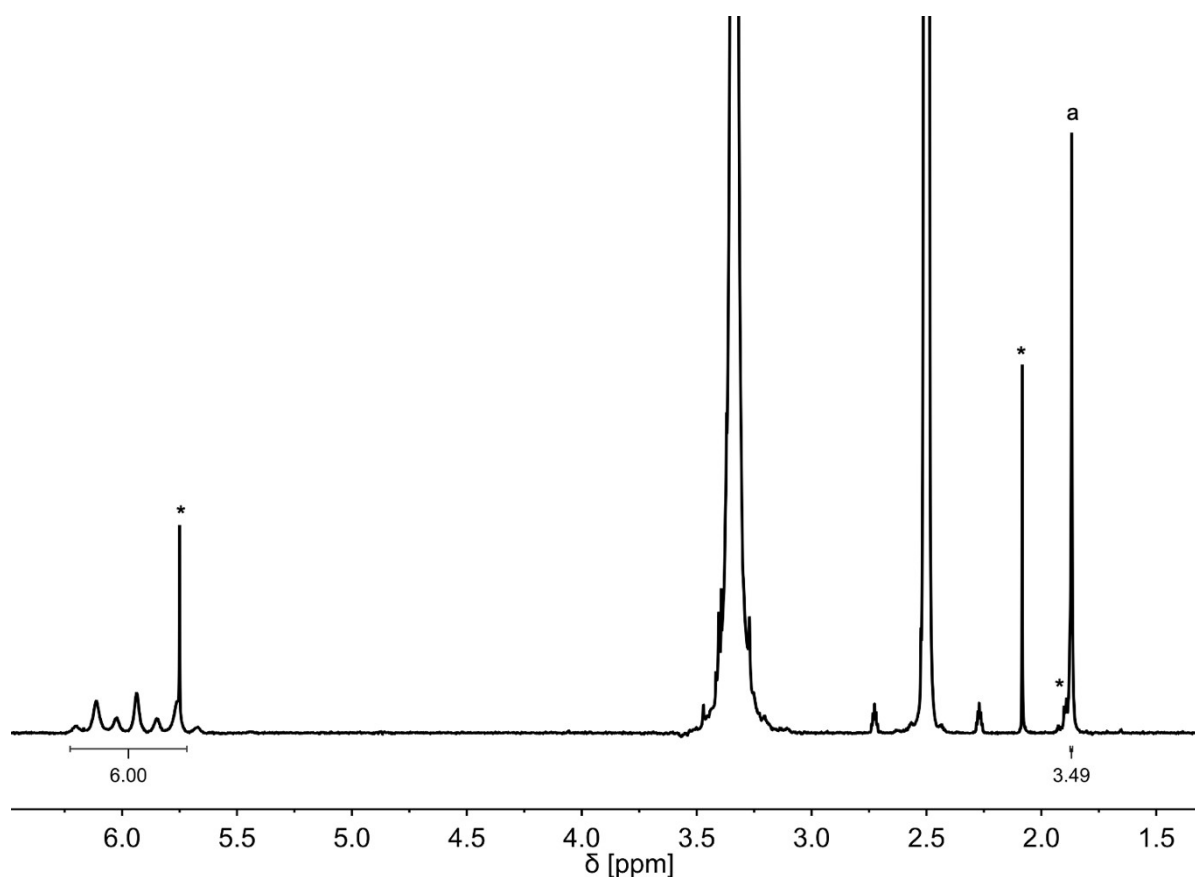
Cisplatin was prepared following the procedure reported by Smith and co-workers.⁷

Step 2. *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(OH)(OCOCH₃)] (IV)

0.20 g of cisplatin were suspended in 97 mL of acetic acid, then 2 mL of hydrogen peroxide were added. The mixture was left stirring at room temperature for 3 hours, protected by light. Progressively, the solution became clear. Next, the reaction mixture was centrifuged (6000 rpm, 3 min) to remove unreacted cisplatin. The supernatant was concentrated under reduced pressure.

An excess of diethyl ether was added to the residue to favor the precipitation of a yellow solid. The product was collected by centrifugation and washed with acetone, dichloromethane, and ether. The final product, IV, was characterized by ¹H-NMR, confirming previously reported assignments.⁸

¹H-NMR (300 MHz, (CD₃)₂SO), δ(ppm): 6.25-5.70 (m, 6H, 2NH₃), 1.87 (s, 3H, H_(a)) (* solvents impurities: 5.75 dichloromethane, 3.33 water, 2.09 acetone, 1.91 acetic acid).

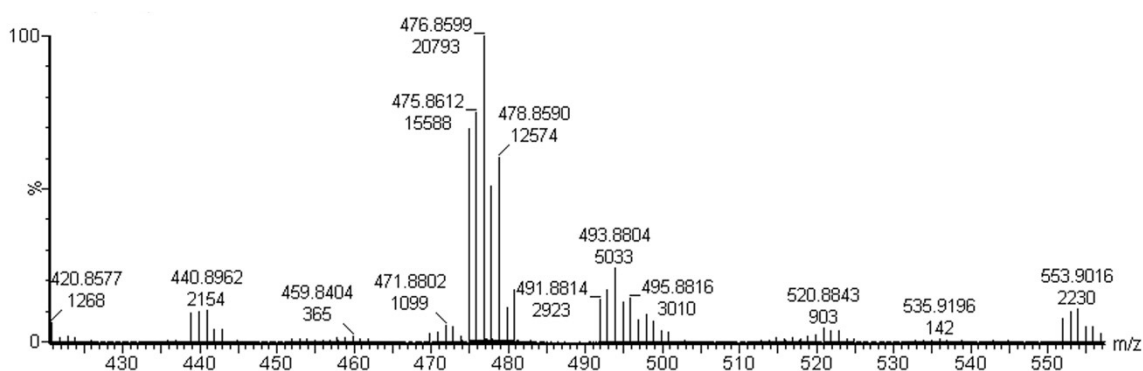
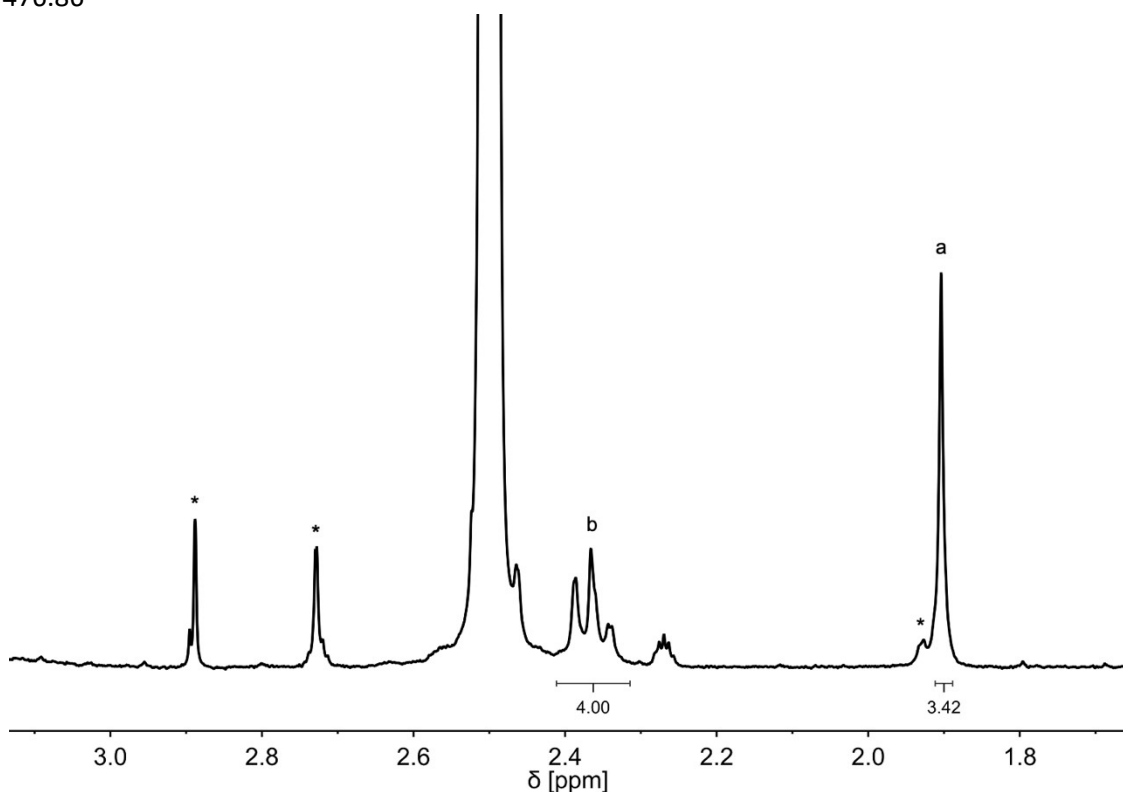


Step 3. *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(OCOCH₃)(O₂CCH₂CH₂CO₂H)] (Pt1)

0.16 g of **IV** were suspended in 1 mL of dry DMF. Succinic anhydride, 0.25 g (6 eq), was then added. The mixture was stirred at 60°C overnight, protected by light.

The day after, 1.5 mL of CH₂Cl₂ and 45 mL of diethyl ether were added to the reaction mixture to form a yellow solid. The mixture was centrifuged (6500 rpm, 3 min) and was dissolved three times with 1 mL of CH₂Cl₂ and 15 mL of diethyl ether. Before the addition of diethyl ether, the product was sonicated until all the product was in suspension. The final product, **Pt1**, was characterized by NMR and ESI-MS as previously reported.⁸

¹H-NMR (300 MHz, (CD₃)₂SO), δ(ppm): 2.42-2.29 (m, 4H, H_(b)), 1.90 (s, 3H, H_(a)) (* solvents impurities: 2.89 and 2.73 dimethylformamide, 1.91 acetic acid). ESI-MS: m/z [M+H]⁺ calculated for C₆H₁₃Cl₂N₂O₆Pt: 475.98, found 476.86

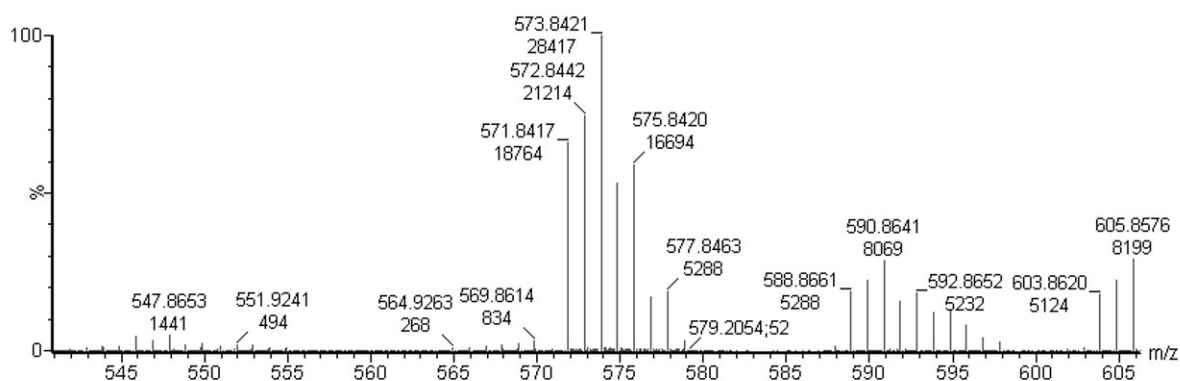
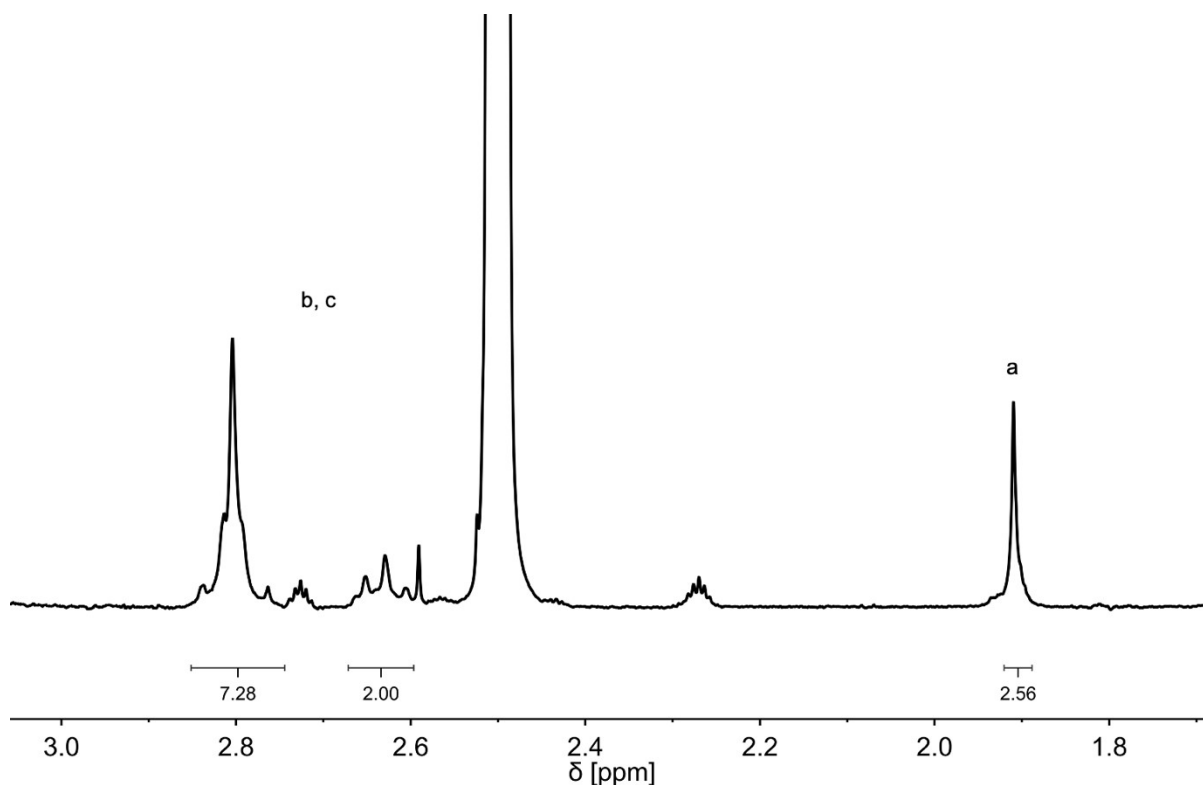


Step 4. Synthesis of N-Hydroxysuccinimide-Activated *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(OCOCH₃)(O₂CCH₂CH₂CO₂H)] (Pt1-NHS)

134 mg of **Pt1** were dissolved in 8 mL of anhydrous acetone. Subsequently, 48.58 mg (1.50 eq) of N-hydroxysuccinimide (NHS) and 70 mg of N,N'-dicyclohexylcarbodiimide (DCC) were added to the mixture which was left stirring overnight at room temperature, protected by light.

The solution was cooled for 1 hour in the freezer and the precipitate was removed by filtration. The product was washed by partial dissolution in acetone (1-2 mL), sonicated and precipitated with diethyl ether (10 mL) four times. The final product, **Pt1-NHS**, was characterized by NMR and ESI-MS confirming literature data.⁸

¹H-NMR (300 MHz, (CD₃)₂SO), δ(ppm): 2.85-2.60 (m, 8H, H_(b), H_(c)), 1.90 (s, 3H, H_(a)). ESI-MS, m/z [M+H]⁺ calculated for C₁₀H₁₇Cl₂N₃O₈Pt: 573.00, found 573.84.



Synthesis of *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(O₂CCH₂CH₂CO₂H)₂] (Pt2)

The Pt2 complex was prepared following the procedure described in Fig. S3. Previously reported methods were adopted for the preparation of cisplatin^{6,7}, V⁹ and Pt2.⁹

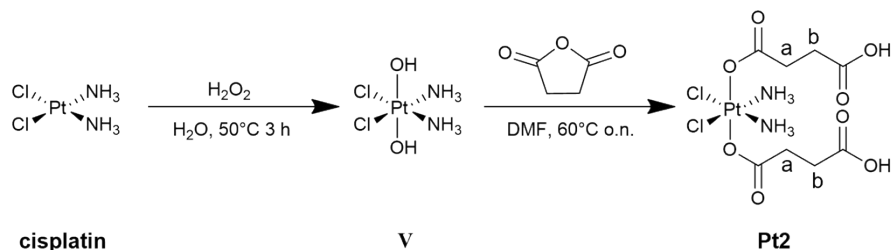


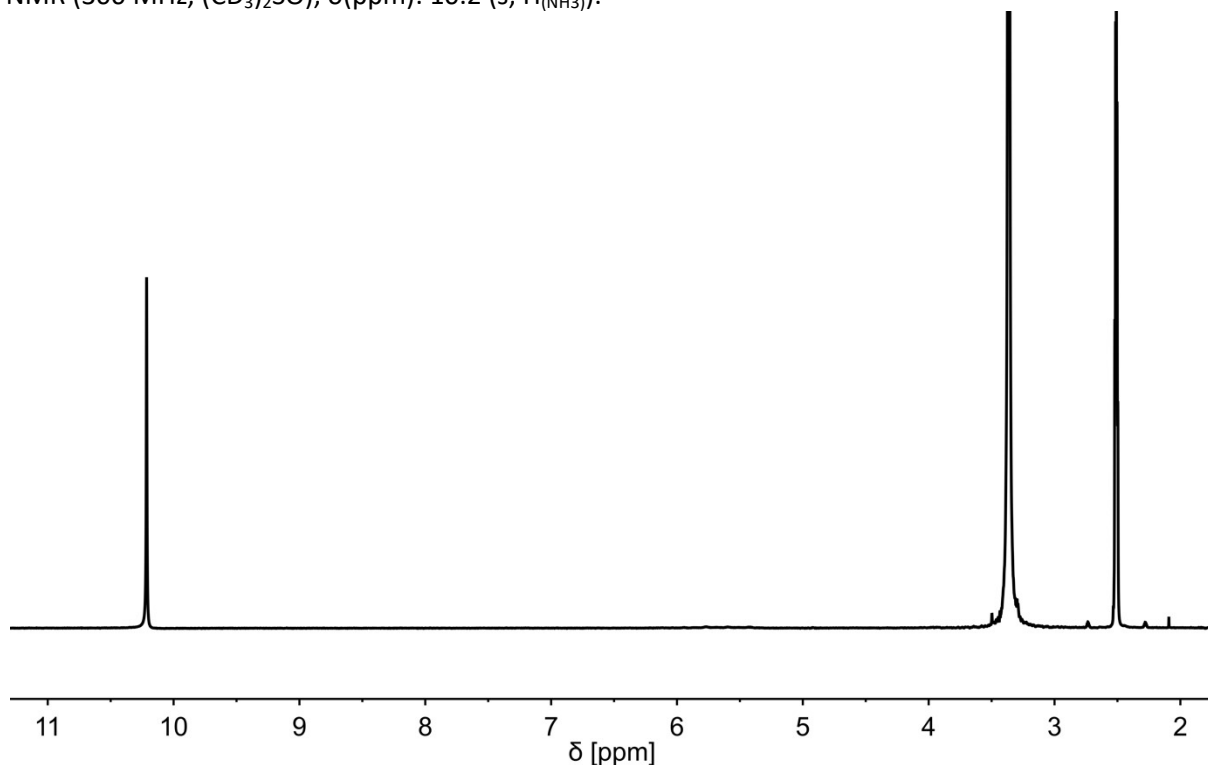
Fig. S3 Synthetic procedure for the preparation of Pt2.

Step 1. *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(OH)₂] (V)

0.18 g of cisplatin were suspended in 1.5 mL of water and then 9 mL of hydrogen peroxide were added. The mixture was left stirring at 50°C for 3 hours, protected by light. Progressively, the solution became clear. Next, the reaction mixture was centrifuged (6000 rpm, 3 min) to remove unreacted cisplatin. The supernatant was concentrated under reduced pressure.

An excess of diethyl ether was added to the residue to favor the precipitation of a yellow solid. The product was collected by centrifugation and washed with acetone, dichloromethane and ether. The final product, V, was characterized by ¹H-NMR, confirming previously reported assignments.⁹

¹H-NMR (300 MHz, (CD₃)₂SO), δ(ppm): 10.2 (s, H_(NH₃)).



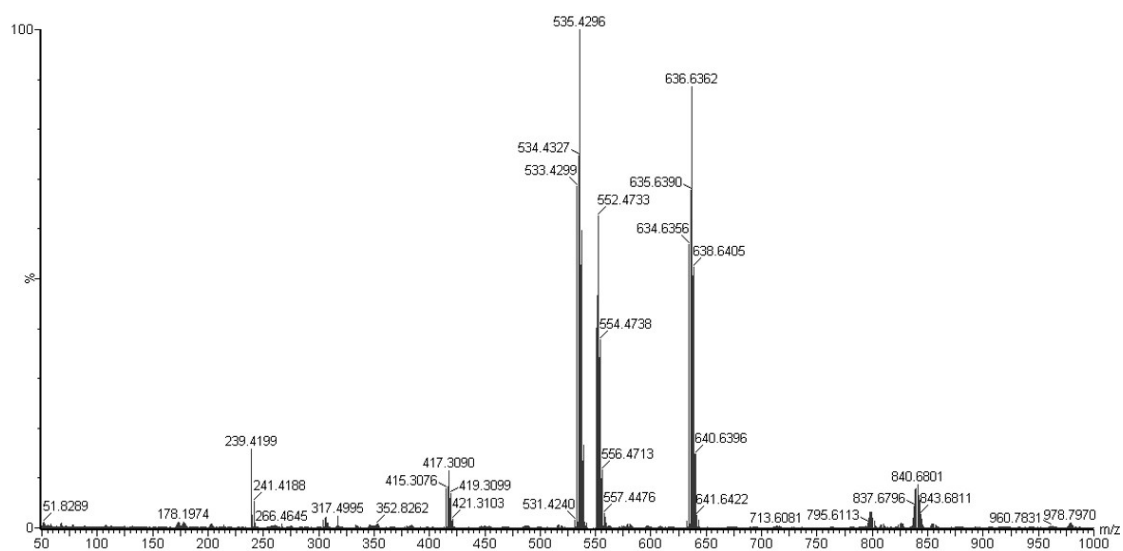
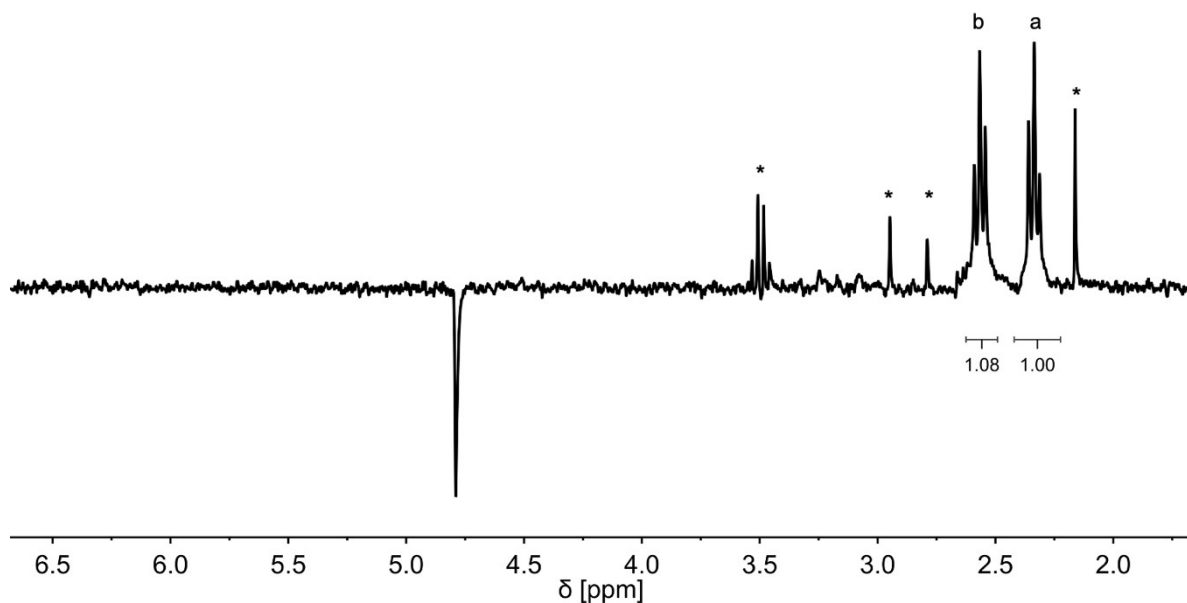
Step 2. *cis,cis,trans*-[Pt(NH₃)₂(Cl)₂(O₂CCH₂CH₂CO₂H)₂] (Pt2)

96.4 mg of V were suspended in 1 mL of dry DMF. Succinic anhydride, 115.7 mg (4 eq.), was then added. The mixture was stirred at 60°C overnight, protected by light.

The day after, 1.5 mL of CH₂Cl₂ and 45 mL of diethyl ether were added to the reaction mixture to form a yellow solid. The mixture was centrifuged (6500 rpm, 3 min) and was dissolved three times with 1 mL of CH₂Cl₂ and 15 mL of diethyl ether. Before the addition of diethyl ether, the product was sonicated until all the

product was in suspension. The final product, **Pt2**, was characterized by NMR and ESI-MS as previously reported.⁹

¹H-NMR (300 MHz, D₂O), δ (ppm): 2.56 (t, J = 6.9 Hz, H_(b)), 2.33 (t, J = 7.0 Hz, H_(a)) (* solvents impurities: 3.56 diethyl ether, 2.94 and 2.80 dimethylformamide, 2.16 acetone). ESI-MS: m/z [M+H]⁺ calculated for C₈H₁₆Cl₂N₂O₈Pt: 534, found 535.43.



3. Protein design and purification

CTPR4/CTPR6/CTPR8. Based on a consensus CTPR4 protein cloned into pPro-EXHTa, two cysteine residues were introduced at positions 17 in the first and third CTPR repeat. The CTPR4 obtained is composed by alternating modules without any mutation, called wild type module (WT, (A E A W Y N L G N A Y Y K Q G D Y D E A I E Y Y Q K A L E L D P R S)), as second and fourth ones, and with two cysteine modules, as first and third ones. The expressed proteins present some additional residues at the N-terminus derived from the cloning strategy and a solvating helix at the C-terminus. The sequence of the amino acids for CTPR4 is reported below. The two cysteines mutation positions are underlined in blue, the His-tag in green and the solvating helix in purple:

CTPR4

```
MSYYHHHHHDYDIPTTENLYFQGAMGS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAKQNLGNAKQKQG
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Based on the mutated CTPR4, previously reported, two and four CTPR(WT) repeats were added to obtain CTPR6 and CTPR8, respectively. The sequences of the amino acids are reported below for CTPR6 and CTPR8, respectively. The two cysteines mutation positions are underlined in blue, the His-tag in green and the solvating helix in purple:

CTPR6

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MSYYHHHHHDYDIPTTENLYFQGAMGS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAKQNLGNAKQKQG
```

CTPR8

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MSYYHHHHHDYDIPTTENLYFQGAMGS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWHNLGHAYYKQGDCDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAWYNLGNAYYKQGDYDEAIEYYQKALELDPRS  
AEAKQNLGNAKQKQG
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All the proteins were expressed as His-tagged fusion and purified first using standard affinity chromatography methods based on previously published protocols¹⁰ for His-tagged CTPR proteins using 0.5% deoxycholic acid in the lysis buffer and secondly by size exclusion chromatography through a HiLoad 16/600 Superdex 75 pg (for CTPR4 and CTPR6) and HiLoad 16/600 Superdex 200 pg (for CTPR8) column using FPLC. The proteins were

then stored in PBS buffer (140 mM NaCl, 50 mM phosphate buffer pH 7.4) with β -mercaptoethanol frozen at -20°C . The protein concentration was determined by UV-absorbance at 280 nm using the extinction coefficient calculated from the amino acid composition ($\epsilon_{\text{CTPR4}} = 54905 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{CTPR6}} = 83660 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{CTPR8}} = 114030 \text{ M}^{-1} \text{ cm}^{-1}$).

4. Bioconjugation of CTPRs

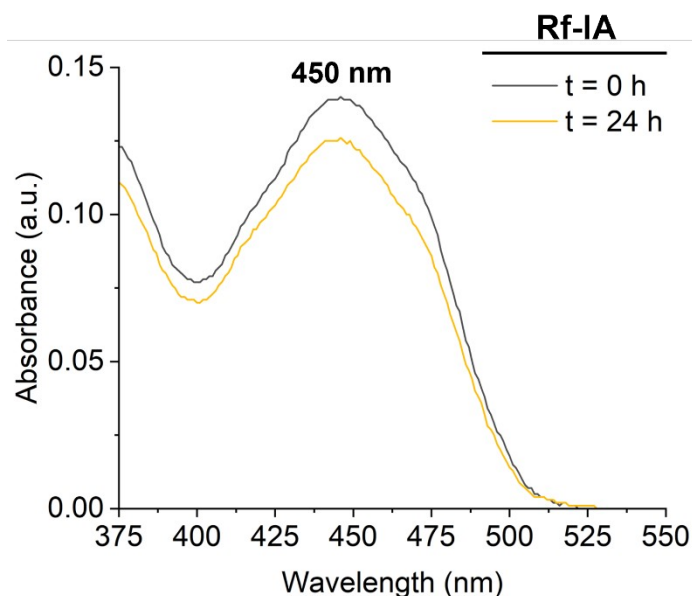


Fig. S4 UV-Vis spectra of **Rf-IA** molecule in PBS buffer (140 mM NaCl, 50 mM phosphate buffer pH 8.5), over 24 hours.

CTPR_n-Rf bioconjugation

We adopted the same procedure for the riboflavin bioconjugation of the three protein scaffolds, namely CTPR4, CTPR6 and CTPR8.¹¹

Prior to any conjugation, purified CTPR at a protein concentration of about 100 μM was freshly reduced with 1 mM 1,4-dithio-DL-treitol (DTT) for 20 minutes to ensure full reduction of the cysteine residues. DTT was removed by buffer exchange over a Nap-5 column (GE Healthcare Life Science, prepacked with Sephadex G-25) in PBS buffer (140 mM NaCl, 50 mM phosphate buffer, pH 8.5). Protein fractions without DTT were eluted directly in the **Rf-IA** solution giving a ratio of 1:5.6 cysteine:**Rf-IA**. The coupling reaction occurred overnight, at room temperature and covered from light. The reaction mixture was then passed through a PD-10 desalting column (GE Healthcare Life Science, prepacked with Sephadex G-25) to eliminate the free dye. The final concentration of CTPR and functionalized riboflavin linked to the protein scaffold was calculated from the absorption spectra taking in account the absorbance correction for the protein's peak at 274 nm. The correction was calculated employing the following equation:

$$A_{274}(\text{corr}) = A_{274} - [A_{450}(\text{dye}) * CF_{274}(\text{dye})]$$

$$CF_{274}(\text{dye}) = A_{274} / A_{450}$$

where A_{274} is the absorbance of the sample at 274 nm, $A_{450}(\text{dye})$ is the absorbance at 450 nm of the functionalized riboflavin, $CF_{274}(\text{dye})$ is the correction factor for the dye at 274 nm (Fig. S5).¹²

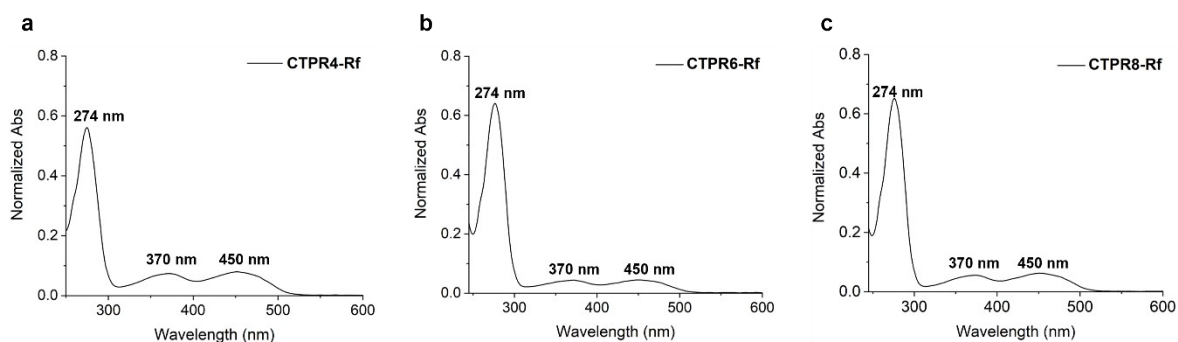


Fig. S5 UV-Vis spectra of conjugated systems: (a) **CTPR4-Rf**, (b) **CTPR6-Rf** and (c) **CTPR8-Rf** in PBS buffer (140 mM NaCl, 50 mM phosphate buffer pH 8.5) after the elimination of free functionalized riboflavin.

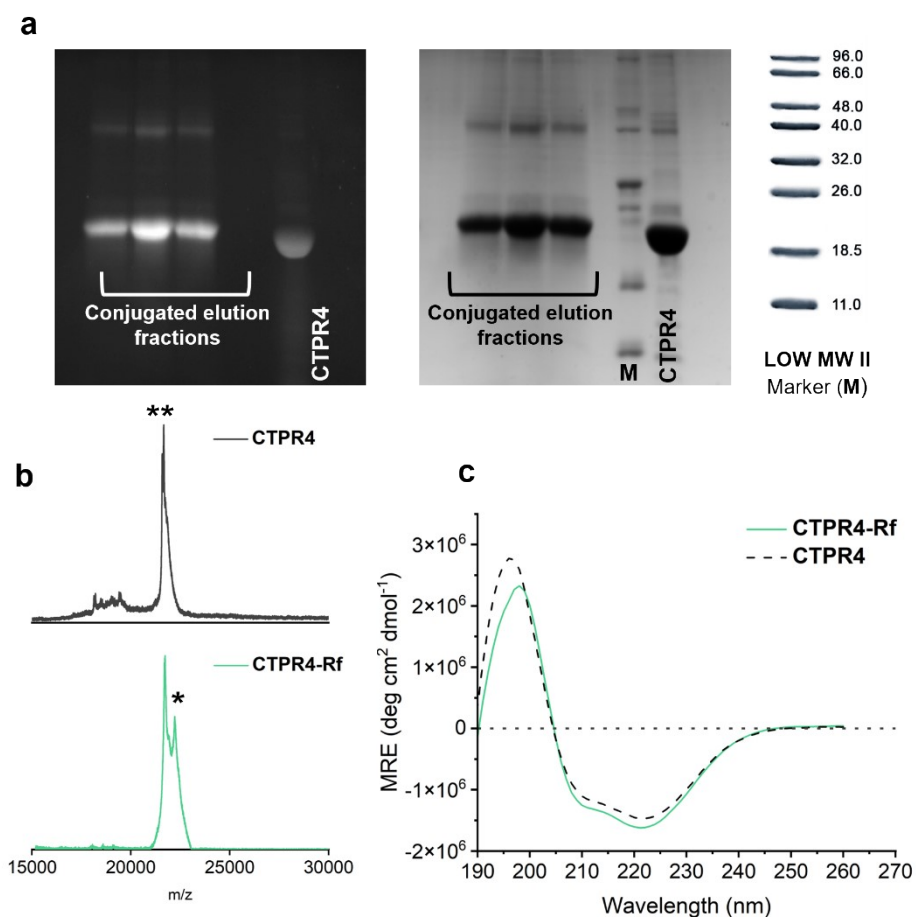


Fig. S6 Characterization of the **CTPR4-Rf** conjugate. (a) SDS-PAGE gels electrophoresis of **CTPR4-Rf** elution volumes, **CTPR4** and marker. The gel is imaged using UV light to monitor the fluorescence of riboflavin (left) and after Coomassie Blue staining (right). (b) MALDI-TOF spectra of **CTPR4-Rf** and **CTPR4** control. Peak assignment: 21.7 kDa **CTPR4**, 22.2 kDa **CTPR4** (21.66 kDa) + **Rf** ($MW_{Rf} = 474$ Da, molecular weight without the leaving group, I). (c) CD spectra in PB buffer of **CTPR4-Rf** (green) and **CTPR4** (black dashed) in the spectral region of the protein secondary structure absorption.

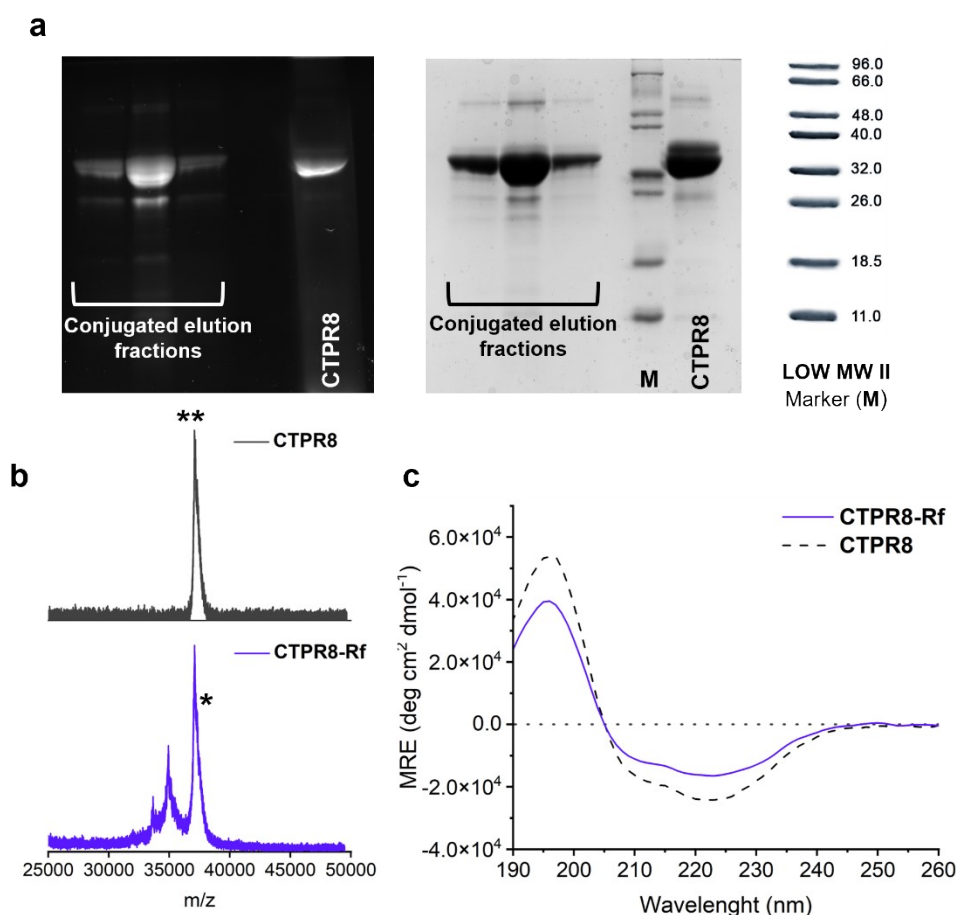


Fig. S7 Characterization of the **CTPR8-Rf** conjugate. (a) SDS-PAGE gels electrophoresis of the **CTPR8-Rf** conjugations elution volumes, CTPR8 alone and marker. The gel is imaged using UV light to monitor the fluorescence of riboflavin (left) and after Coomassie Blue staining (right). (b) MALDI-TOF spectra of **CTPR8-Rf** and **CTPR8** alone (control). Peak assignment: 37.0 kDa **CTPR8**, 37.4 kDa **CTPR8** (37.0 kDa) + **Rf** ($MW_{Rf} = 474$ Da, molecular weight without the leaving group, I). (c) CD spectra in PB buffer of **CTPR8-Rf** conjugate (purple) and CTPR alone (dash) in the spectral region of the protein secondary structure absorption.

CTPR6-Rf-Pt1 bioconjugation

CTPR6-Rf conjugated samples were reacted with the NHS-activated Pt complex (**Pt1-NHS**). To a 25 μ M CTPR6(2Cys), 10 eq of **Pt1-NHS** (stock solution: 4.6 mM in PBS buffer 140 mM NaCl, 50 mM phosphate buffer, pH 7.4) were added using a lysine:Pt ratio of 1:10. The reaction was carried out overnight, at room temperature and covered from light. The reaction mixture was passed through a PD-10 desalting column (GE Healthcare Life Science, prepacked with Sephadex G-25) to eliminate unreacted **Pt1-NHS** complex. The final concentration of metal complex linked to CTPR6 scaffold was calculated with ICP-MS resulting in a ratio of 1:5 CTPR6:Pt. *Note:* conjugation reaction of 2 h should be optimal based on tests performed at a later stage monitoring the stability of **Pt1-NHS** in PBS buffer (pH 7.4) overtime.

CTPR6-Pt1 bioconjugation

To a 25 μ M CTPR6, 10 eq of **Pt1-NHS** (stock solution: 4.6 mM in PBS buffer, 140 mM NaCl, 50 mM phosphate buffer, pH 7.4) were added using a lysine:Pt ratio of 1:10. The reaction occurred overnight, at room temperature and covered by light. The reaction mixture was passed through a PD-10 desalting column (GE Healthcare Life Science, prepacked with Sephadex G-25) to eliminate free **Pt1-NHS** complex. The final concentration of metal complex linked to the CTPR6 scaffold was calculated by ICP-MS mass spectrometry measurements resulting in a ratio of 1:5 CTPR6:Pt. *Note:* conjugation reaction of 2 h should be optimal based on tests performed at a later stage monitoring the stability of **Pt1-NHS** in PBS buffer (pH 7.4) overtime.

5. Photostability

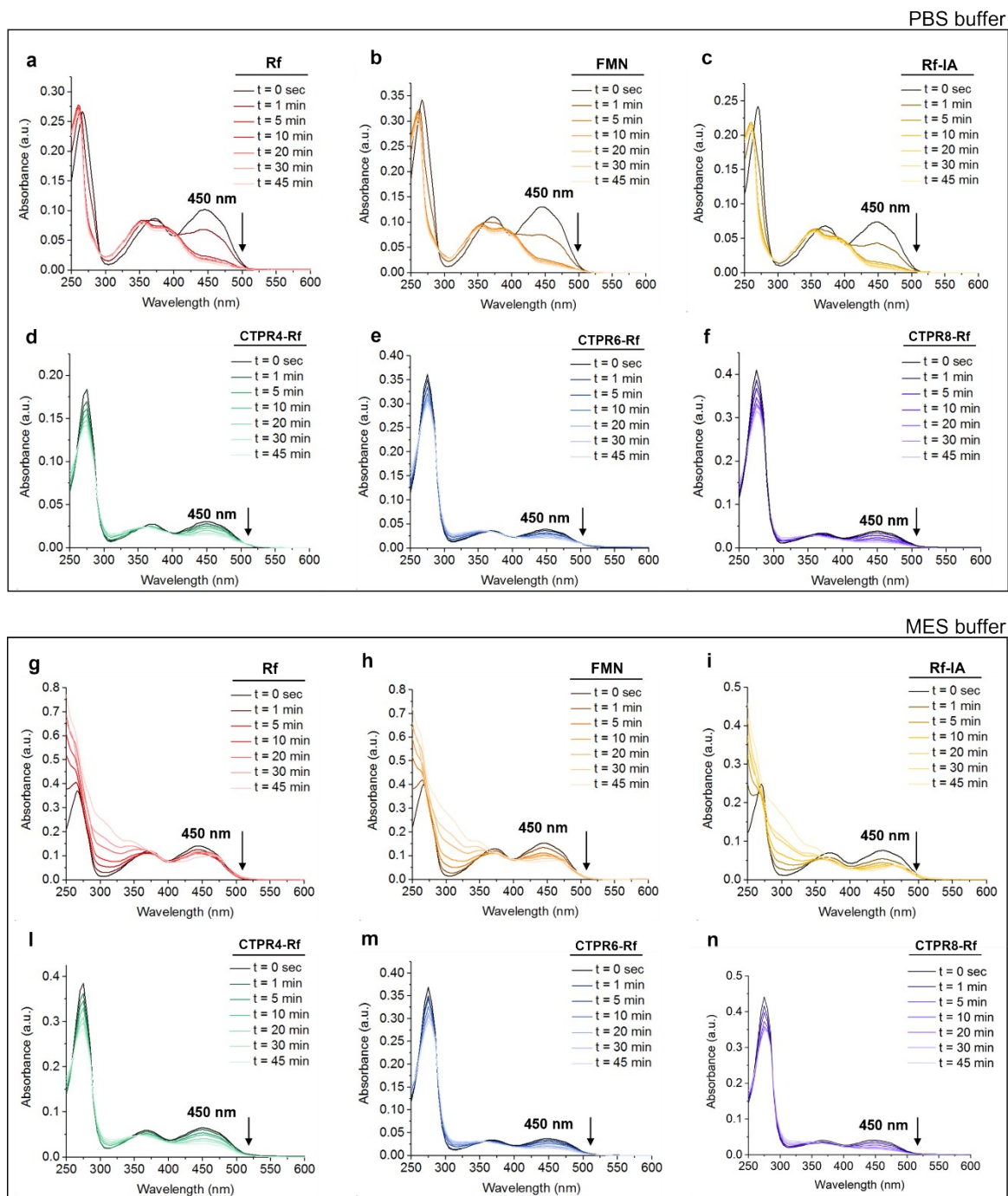
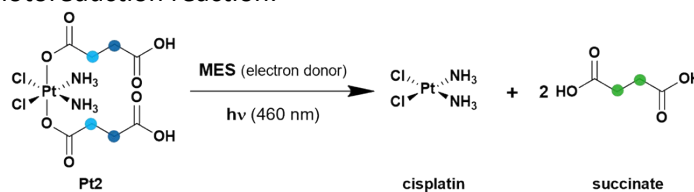


Fig. S8 Photostability of Rf, FMN, Rf-IA and CTPR_n-Rf in (a-f) PBS (140 mM NaCl, 50 mM phosphate buffer pH 7.4) and (g-n) MES buffer (18 mM, pH 6.4) under blue light irradiation (460 nm, 3.5 mW/cm²).

6. Photocatalysis studies

Photocatalysis experiments

All photocatalysis experiments were monitored following the formation of the singlet signal, related to the release of the succinate for the reduction of the platinum complex. Here below is reported a schematic representation of **Pt2** photoreduction reaction:



Photocatalysis tests were carried in a NMR tube with a total volume of 500 μL with the following composition: 500 μM platinum prodrug, 5 μM flavin catalyst, 7 μM and 12 μM substrate, 18 mM MES (pH 6.4) in 10% D_2O - 90% H_2O . Samples were irradiated with a custom-made array of blue light emitting diodes (460 nm, 3.5 mW/cm^2).

Integration of the free succinate ligand signal (2.25–2.35 ppm) was used for monitoring the reaction progress.

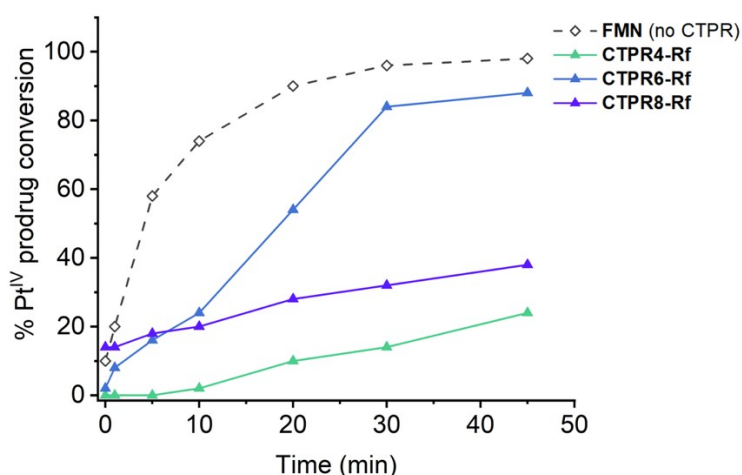


Fig. S9 Photocatalytic activation of free **Pt2** by **FMN**, **CTPR4-Rf**, **CTPR6-Rf** and **CTPR8-Rf**. Experimental conditions: [**Pt2**] = 500 μM , [**CTPR_n-Rf**]_{Rf} = 5 μM , [**FMN**] = 5 μM , [**MES**] = 18 mM (pH 6.4), $h\nu = 460 \text{ nm}$ (3.5 mW/cm^2).

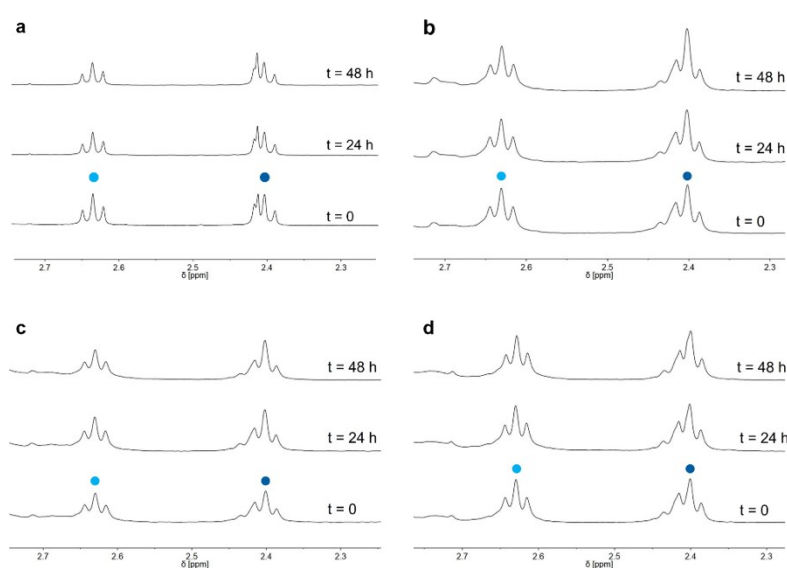


Fig. S10 Dark stability of **Pt2** in the presence of **FMN** and the protein-catalyst bioconjugate systems in MES buffer (10% D_2O): (a) **FMN**, (b) **CTPR4-Rf**, (c) **CTPR6-Rf**, (d) **CTPR8-Rf**.

Experimental conditions: [**Pt2**] = 500 μM , [**FMN**] = 5 μM , [**CTPR_n-Rf**]_{Rf} = 5 μM , [**CTPR₄-Rf**]_{CTPR4} = 7.6 μM , [**CTPR₆-Rf**]_{CTPR6} = 12 μM , [**CTPR₈-Rf**]_{CTPR8} = 7.7 μM , [**MES**] = 18 mM (pH 6.4), $h\nu = 460 \text{ nm}$ (3.5 mW).

$^1\text{H-NMR}$ signal labeling: ● Pt-OCOCH₂CH₂CO₂H, ● Pt-OCOCH₂CH₂CO₂H, and ● free O₂CCH₂CH₂CO₂H.

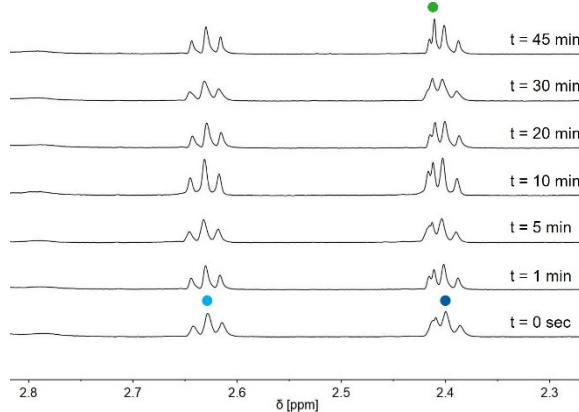


Fig. S11 Photocatalytic activation of **Pt2** complex in MES buffer (10% D₂O). Experimental conditions: [**Pt2**] = 500 μM, [MES] = 18 mM (pH 6.4), $h\nu$ = 460 nm (3.5 mW/cm²).

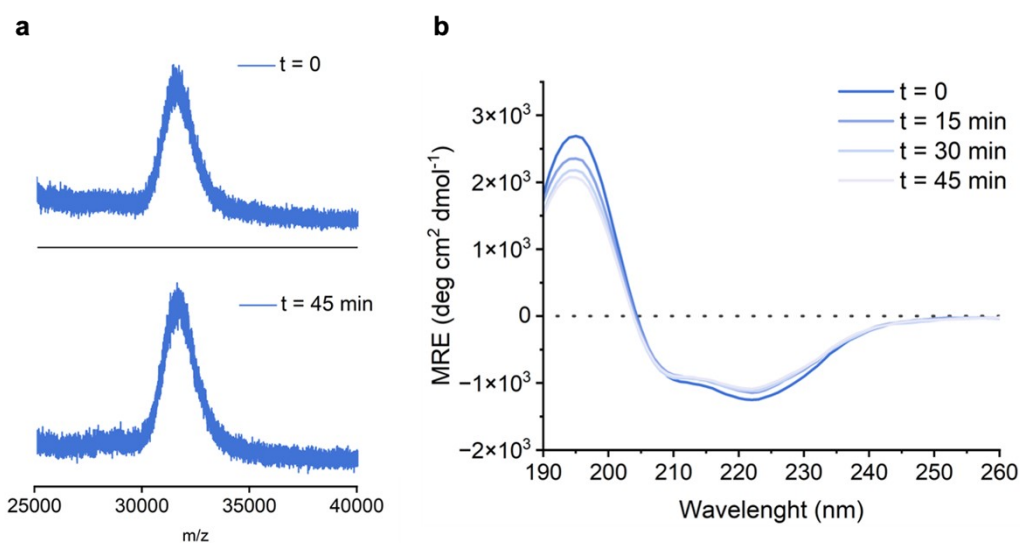


Fig. S12 Photostability of **CTPR6-Rf-Pt1**. (a) MALDI-TOF spectra of **CTPR6-Rf-Pt1** before ($t = 0$) and after 45 min of light irradiation (460 nm, 3.5 mW/cm²). (b) CD spectra in PB buffer of **CTPR6-Rf-Pt1** at different irradiation times (460 nm, 3.5 mW/cm²) in the spectral region of the protein secondary structure absorption.

7. Incubation experiments with GSH

The dark stability of **Pt1** and **CTPR6-Rf-Pt1** in the presence of the biological thiol L-glutathione (GSH) was investigated in comparison to cisplatin. **Pt1**, **CTPR6-Rf-Pt1**, and cisplatin (50 μM Pt) were incubated for 24 h with an excess of GSH (1:100) in aqueous solution at room temperature. The samples were monitored at three different time points: 0, 6 and 24 h using UPLC-MS.

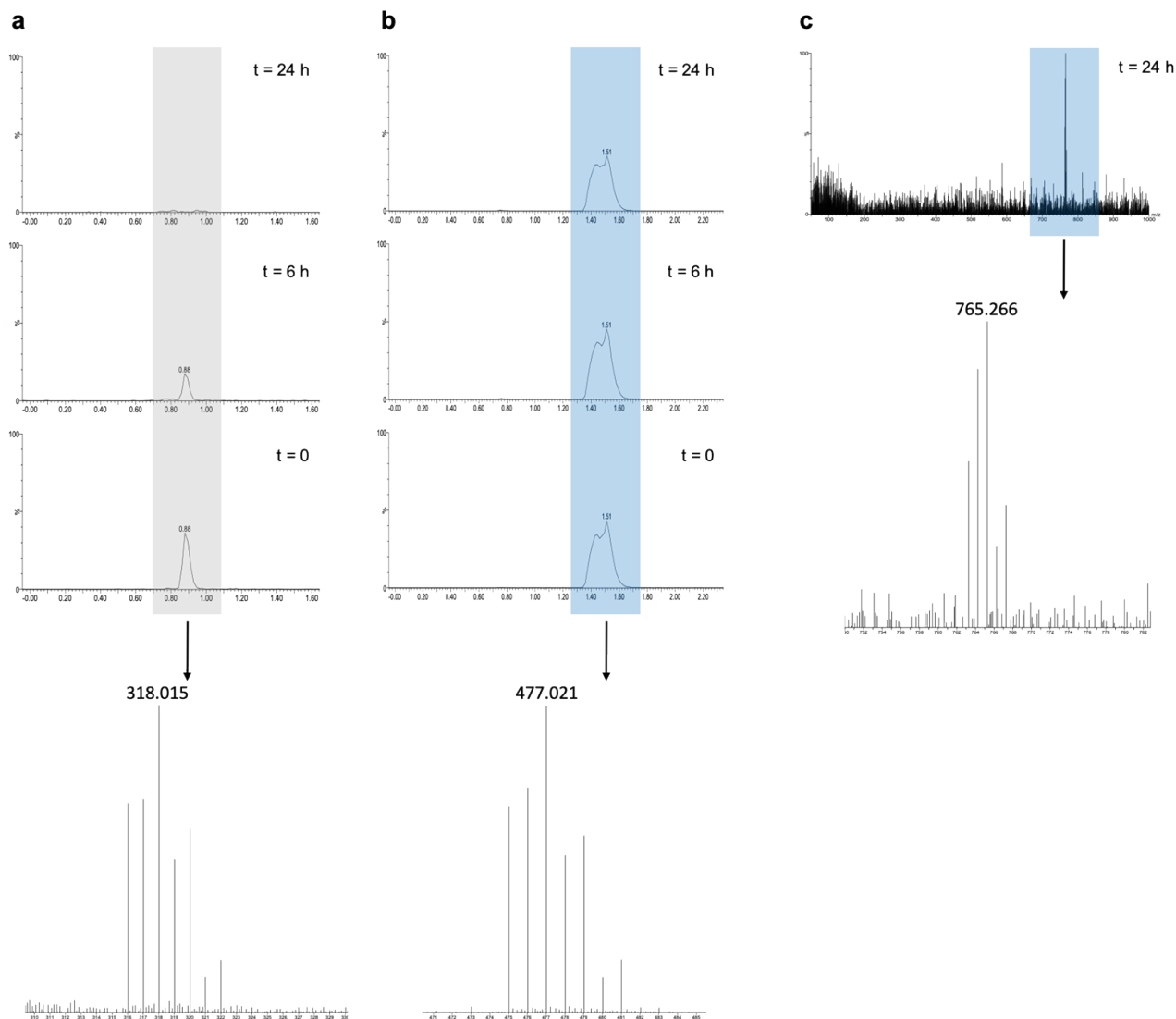


Fig. S13 (a) Dark stability of cisplatin (50 μM) co-incubated with GSH (500 μM) in water, monitored at 0, 6 h and 24 h: (top) UPLC chromatogram, (bottom) mass spectrum of the elution peak at 0.88 min ($m/z = 318.015$), corresponding to $[\text{Pt}(\text{Cl})_2(\text{NH}_3)_2 + \text{NH}_4^+]$; (b) Dark stability of **Pt1** (50 μM) co-incubated with GSH (500 μM), monitored at 0, 6 h and 24 h: (top) UPLC chromatogram, (bottom) mass spectrum of the elution peak at 1.56 min ($m/z = 477.021$), corresponding to $[\text{Pt1} + \text{H}^+]$; (c) UPLC chromatograms for **CTPR6-Rf-Pt1** (50 μM) co-incubated with GSH (500 μM) at 0, 6 h and 24 h did not show any major MS peak corresponding to free Pt species. We could detect traces of a Pt species ($m/z = 765.266$) only for the 4.2 min elution peak after 24 h of incubation with GSH.

8. Cell Viability experiments

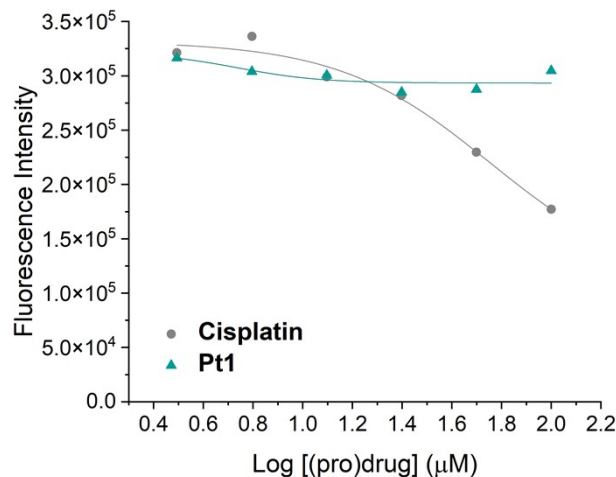


Fig. S14 Dose response curve of cisplatin and **Pt1** complex in PANC-1 cancer cell line. Experimental conditions for both metals: 3.12 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM. 24 hours uptake in dark, 37°C.

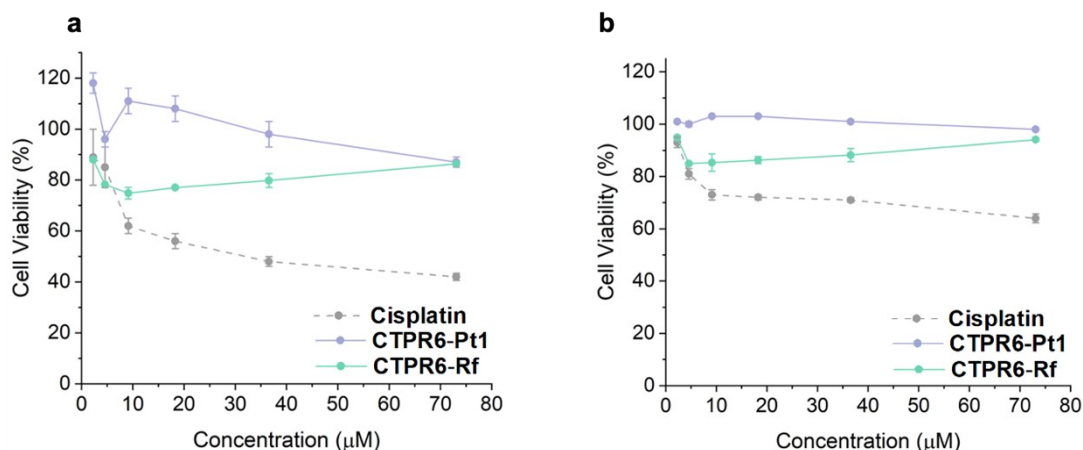


Fig. S15 Percentage cell viability in PANC-1 cancer cells following treatment with cisplatin (dashed line), **CTPR6-Pt1** (violet) and **CTPR6-Rf** (green) systems (a) under light irradiation (45 min, on/off regime 460 nm, 3.5 mW/cm²) and (b) in dark conditions. The concentrations reported are related to the ones of drug/prodrug.

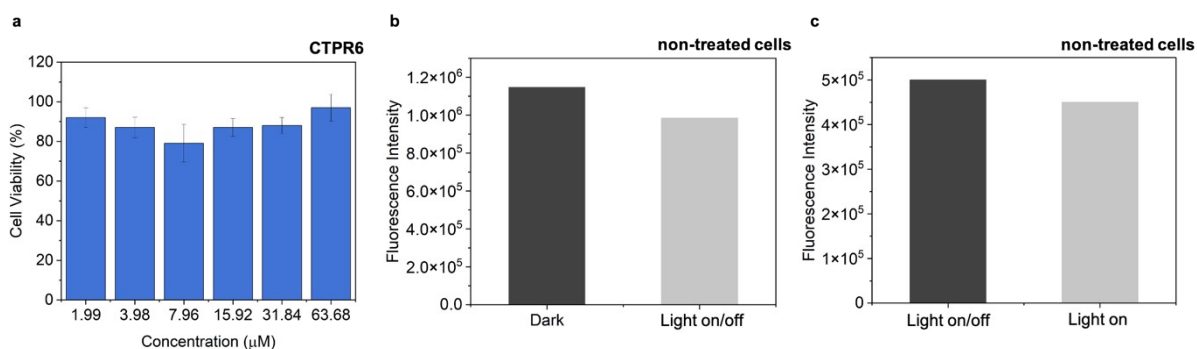


Fig. S16 (a) Cell viability percentage in PANC-1 cancer cells following treatment with **CTPR6** in dark condition. Experimental conditions: [CTPR6] = 1.99 μM, 3.98 μM, 7.96 μM, 15.92 μM, 31.84 μM, 63.68 μM. 24 hours uptake in dark, 37°C. (b) Fluorescence intensity of non-treated PANC-1 cancer cells in dark condition (dark gray) and under light on/off (light gray). The cells were irradiated for 45 min total, with blue light (3.5 mW/cm²). (c) Fluorescence intensity of non-treated PANC-1 cancer cells with light on/off (dark gray) and with light on (light gray). The cells were irradiated for 45 min total, with blue light (3.5 mW/cm²).

References

- 1 H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **62**, 7512–7515.
- 2 J. B. Metternich, S. Sagebiel, A. Lückener, S. Lamping, B. J. Ravoo and R. Gilmour, *Chem. Eur. J.*, 2018, **24**, 4228–4233.
- 3 A. Y.-T. Huang, C.-H. Tsai, H.-Y. Chen, H.-T. Chen, C.-Y. Lu, Y.-T. Lin and C.-L. Kao, *Chem. Commun.*, 2013, **49**, 5784.
- 4 J. Alguacil, S. Defaus, A. Claudio, A. Trapote, M. Masides and J. Robles, *Eur. J. Org. Chem.*, 2010, **2010**, 3102–3109.
- 5 C. Garino, S. Ghiani, R. Gobetto, C. Nervi, L. Salassa, V. Ancarani, P. Neyroz, L. Franklin, J. B. Alexander Ross and E. Seibert, *Inorg. Chem.*, 2005, **44**, 3875–3879.
- 6 R. A. Alderden, M. D. Hall and T. W. Hambley, *J. Chem. Educ.*, 2006, **83**, 728.
- 7 K. M. Brière, R. Goel, F. H. Shirazi, D. J. Stewart and I. C. P. Smith, *Cancer Chemother. Pharmacol.*, 1996, **37**, 518–524.
- 8 H. Yao, Z. Xu, C. Li, M.-K. Tse, Z. Tong and G. Zhu, *Inorg. Chem.*, 2019, **58**, 11076–11084.
- 9 M. Ravera, E. Perin, E. Gabano, I. Zanellato, G. Panzarasa, K. Sparnacci, M. Laus and D. Osella, *J. Inorg. Biochem.*, 2015, **151**, 132–142.
- 10 T. Kajander, A. L. Cortajarena and L. Regan, in *Protein Design*, Humana Press, New Jersey, 2006, vol. 340, pp. 151–170.
- 11 S. H. Mejías, J. López-Andarias, T. Sakurai, S. Yoneda, K. P. Erazo, S. Seki, C. Atienza, N. Martín and A. L. Cortajarena, *Chem. Sci.*, 2016, **7**, 4842–4847.
- 12 L. De Rosa, A. L. Cortajarena, A. Romanelli, L. Regan and L. D. D’Andrea, *Org. Biomol. Chem.*, 2012, **10**, 273–280.