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SUPPORTING INFORMATION

Unexpected photoactivation pathways in a folate-receptor-targeted *trans*-diazido Pt(IV) anticancer pro-drug.

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1.- Materials and Methods

Unless otherwise stated, common chemicals and solvents (HPLC grade or reagent grade quality) were purchased from commercial sources and used without further purification. Aluminium plates coated with a 0.2 mm thick layer of silica gel 60 F_{254} were used for thin-layer chromatography analyses (TLC), whereas flash column chromatography purification was carried out using silica gel 60 (230-400 mesh). Reversed-phase high-performance liquid chromatography (HPLC) analyses were carried out on a Jupiter Proteo C_{18} column (250x4.6 mm, 90 Å 4 μm, flow rate: 1 mL/min) using linear gradients of 0.1% formic acid in H_2O (A) and 0.1% formic acid in ACN (B). NMR spectra were recorded at 25 ^{o}C in a 400 MHz spectrometer using the deuterated solvent as an internal deuterium lock. The residual protic signal of DMSO was used as a reference in ^{1}H and ^{13}C NMR spectra recorded in DMSO-d_6. Chemical shifts are reported in part per million (ppm) in the δ scale, coupling constants in Hz and multiplicity as follows: s (singlet), d (doublet), t (triplet), q (quartet), qt (quintuplet), m (multiplet), dd (doublet of doublets), dq (doublet of quartets), br (broad signal), etc. Electrospray ionization mass spectra (ESI-MS) were recorded on an instrument equipped with single quadrupole detector coupled to an HPLC, and high-resolution (HR) ESI-MS on a LC/MS-TOF instrument.

Analysis of platinum content was carried out on ICP-OES 5300DV (Perkin Elmer) or ICPMS 7500cx (Agilent) instruments. The emission wavelength used for Pt ICP-OES detection was 265.945 nm, and ^{195}Pt was detected in ICP-MS using ^{166}Er (50 ppb) as an internal standard.

2.- Synthesis and characterization of Pt-folate conjugate (3)

Caution! No problem was encountered during the work reported here, but due care and attention with appropriate precautions should be taken in the synthesis and handling of heavy metal azides. All syntheses and purifications were carried out in the dark with minimal light exposure.

To a solution of *trans,trans,trans*-[$Pt(N_3)_2(OH)(succ)(py)_2$] (**2**) (4.68 mg, 8.27 μmol) and HATU (3.12 mg, 8.27 μmol) in anhydrous DMF (1 mL) under an Ar atmosphere, DIPEA (13 μL, 74 μmol) was added and the mixture stirred for 10 min under Ar at room temperature and protected from light. After addition of a solution of folic acid derivative **4**[1] (4.8 mg, 7.40 μmol) and DIPEA (13 μL, 74 μmol) in anhydrous DMF (1 mL), the reaction mixture was stirred for 2 h at room temperature under Ar and protected from light. After evaporation under reduced pressure, the crude material was dissolved in 1:1 (v/v) mixture of H_2O and ACN and lyophilized. As shown in Figure S1, analytical reversed-phase HPLC analysis (0−50% B in 30 min; A, 0.036% TFA in H_2O; B, 0.045% TFA in ACN; flow rate, 1 mL/min) revealed the presence of a main peak that was characterized as the expected Pt-folate conjugate **3** (R_t = 23.1 min). The solution was lyophilized, and the conjugate was purified by semipreparative RP-HPLC (gradient from 0−80% B in 30 min; A, 0.1% TFA in H_2O; B, 0.1% TFA in ACN; flow rate, 3 mL/ min; R_t = 16.9 min). Overall yield (synthesis + purification): 2.87 mg of a yellow solid, 32%. ^{1}H NMR (400 MHz, DMSO-d_6, δ (ppm)): 11.46 (1H, s), 8.80 (4H, m), 8.64 (1H, s), 8.27 (2H, m), 8.15 (1H, m), 7.82 (4H, m), 7.71 (1H, t, *J* = 5.4 Hz), 7.64 (2H, d, *J* = 8.6 Hz), 6.92 (1H, t, *J* = 6.1 Hz), 6.63 (2H, d, *J* = 8.6 Hz), 4.48 (2H, d, *J* = 6.0 Hz), 4.25 (1H, m), 3.48 (4H, m), 3.44 (4H, m), 3.35 (4H, m), 3.06 (4H, m), 2.44 (2H, t, *J* = 7.4 Hz), 2.22 (2H, t, *J* = 7.4 Hz), 2.16 (2H, m), 2.03 (1H, m), 1.89 (1H, m), 1.58 (4H, m). ^{13}C NMR (101 MHz, DMSO-d_6, δ (ppm)): 174.8, 173.8, 172.9, 171.5, 171.3, 150.7, 149.3, 148.7, 141.9, 128.9, 127.9, 126.2, 121.4, 111.2, 69.7, 69.5, 69.3, 68.1, 68.0, 65.5, 62.6, 45.9, 35.8, 33.5, 32.0, 31.9, 31.7, 31.3, 29.4, 29.3, 29.0, 28.9, 28.7, 28.5, 24.4, 22.1, 14.0. HRMS (ESI-TOF, negative mode) (*m/z*): $[M-H]^-$ Calcd for $C_{43}H_{54}N_{17}O_{12}Pt$, 1195.3786; found, 1195.3773; $[M-2H]^{2-}$ Calcd for $C_{43}H_{53}N_{17}O_{12}Pt$, 597.1854; found, 597.1870. Analytical HPLC (10−70% B in 30 min, formic acid additive): R_t = 17.5 min.

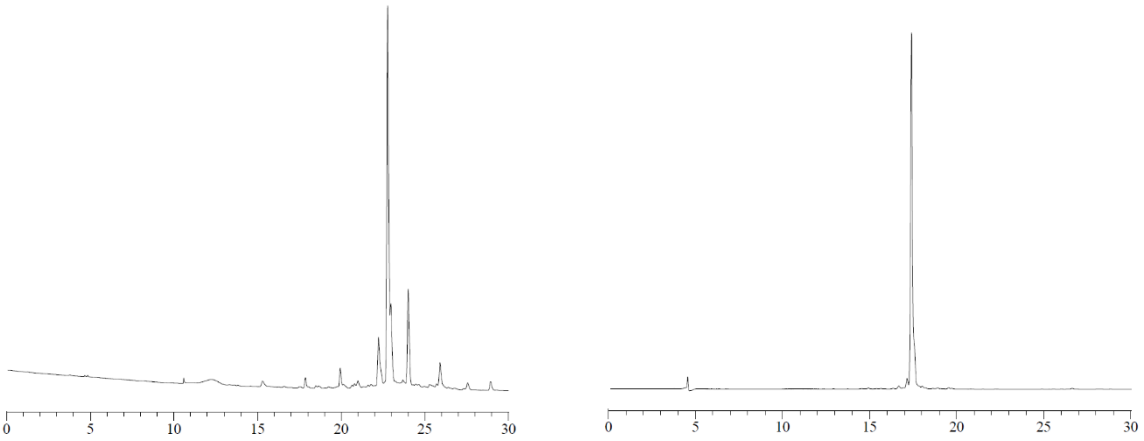

Figure S1. Reversed-phase HPLC traces (260 nm detection) of Pt-folate conjugate **3**, reaction crude (left) and purified (right).

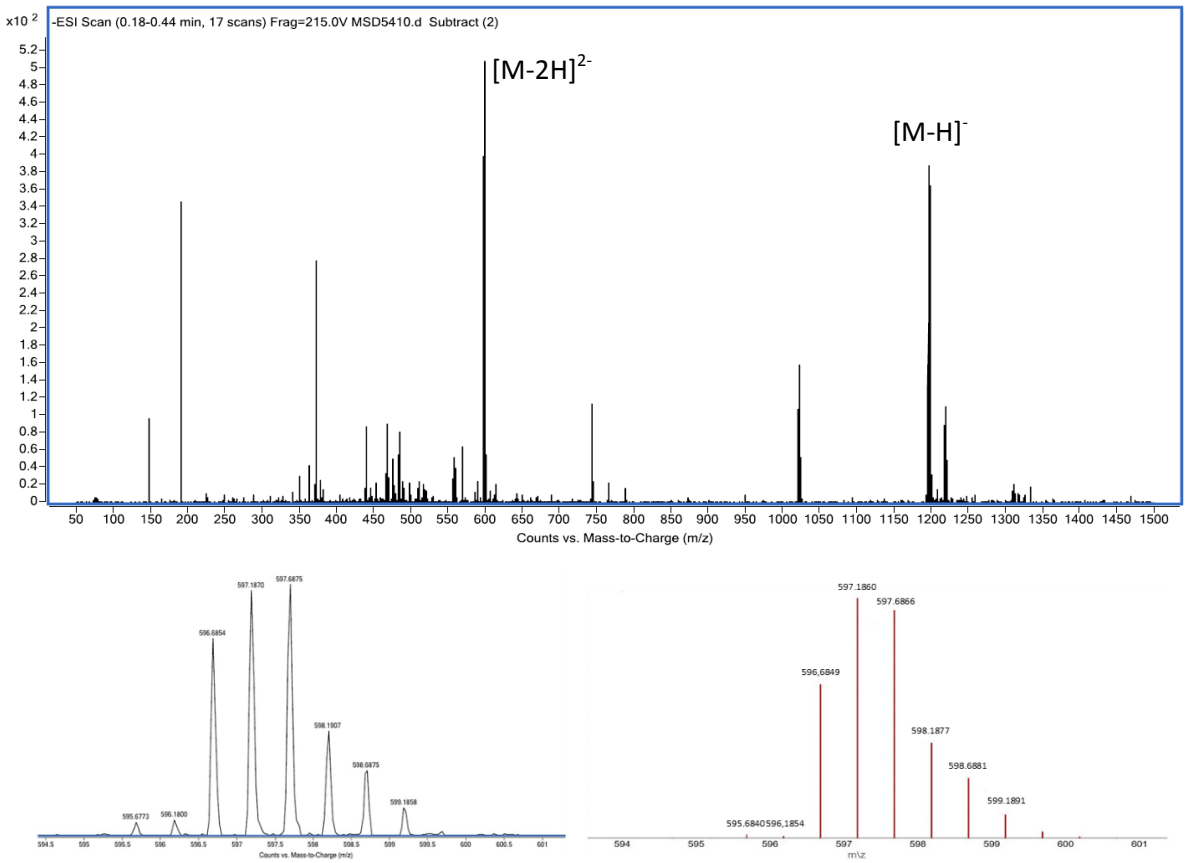

Figure S2. HR-ESI-MS (negative mode) of Pt-folate conjugate **3** and expanded mass spectrum of the molecular peak of **3** ($[M - 2H]^{2-}$), experimental (left) and calculated (right).

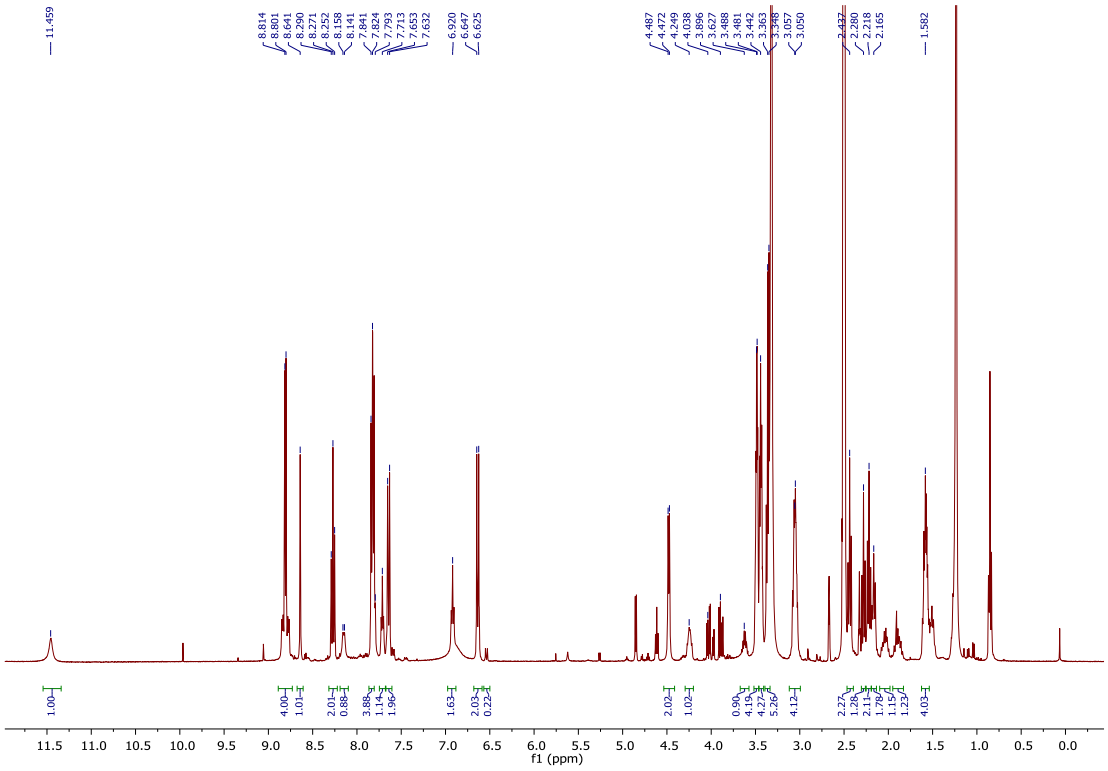

Figure S3. ^{1}H NMR spectrum of Pt-folate conjugate **3** in DMSO-d_6.

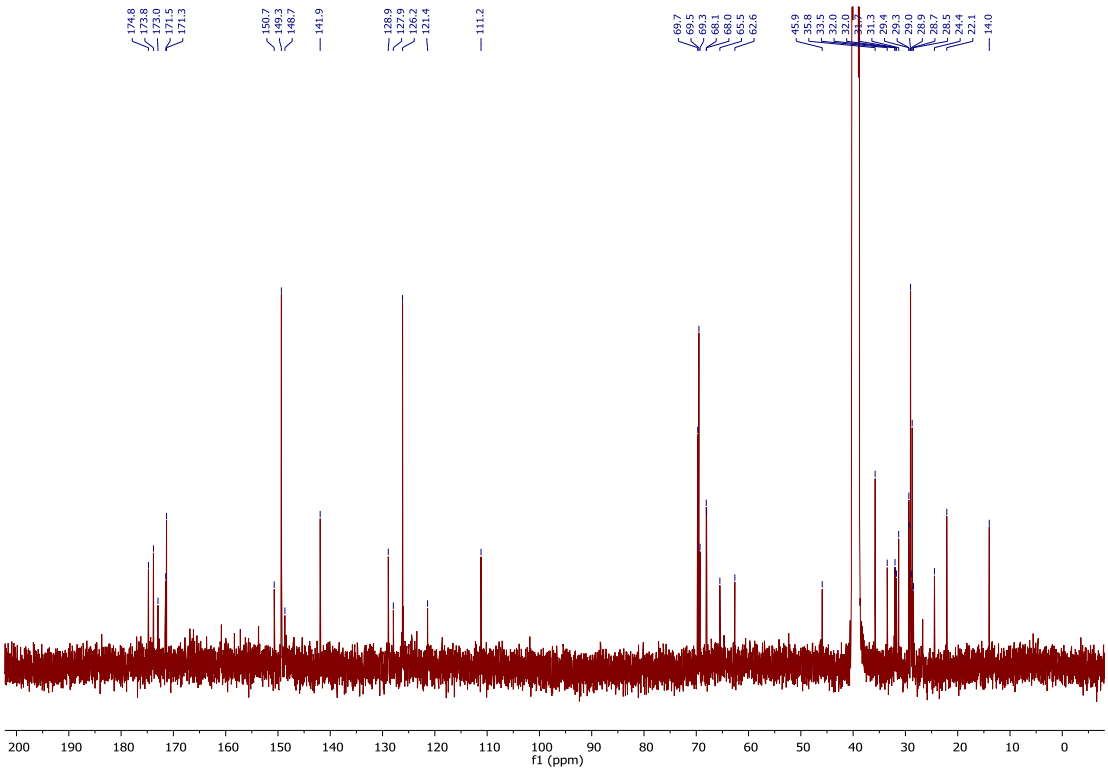

Figure S4. ^{13}C NMR spectrum of Pt-folate conjugate **3** in DMSO-d_6.

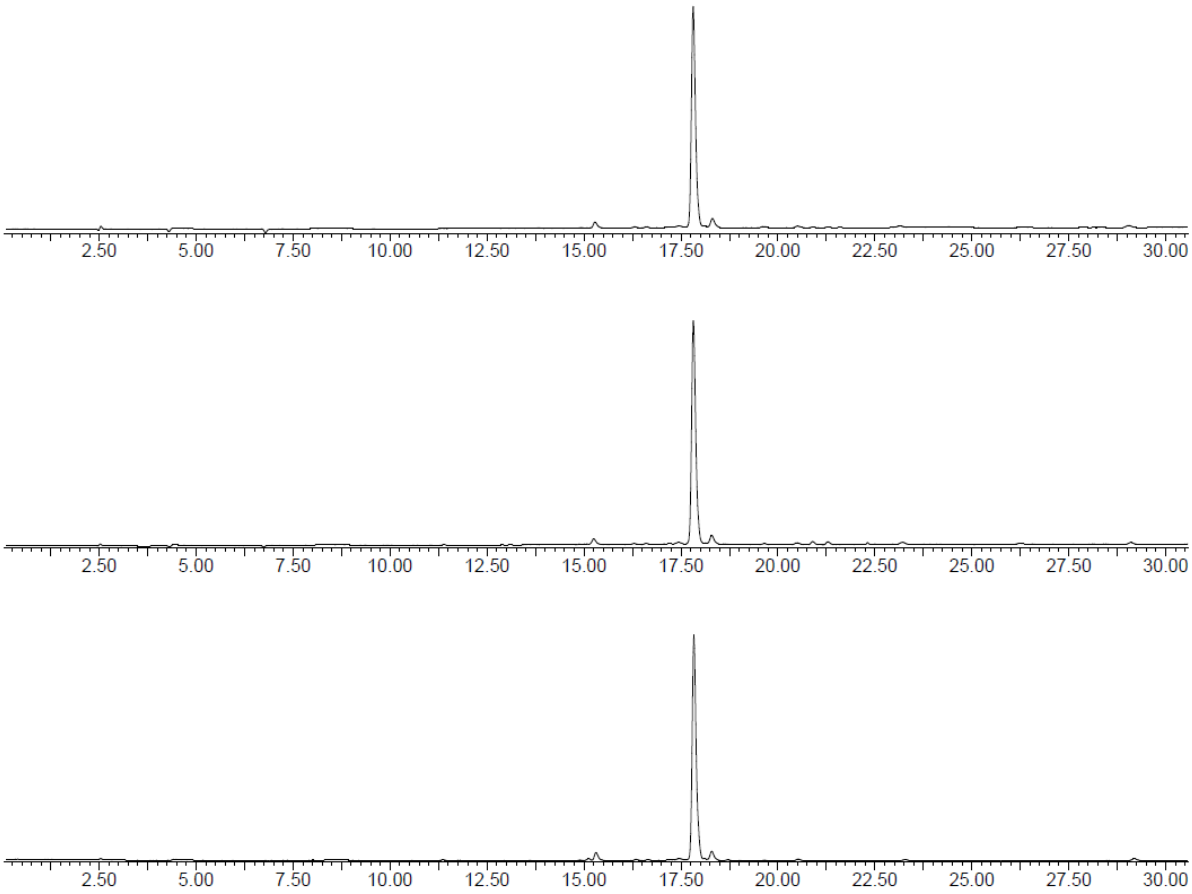

Figure S5. Stability of Pt-folate conjugate **3** in PBS buffer in the dark at 310 K. Reversed-phase HPLC traces (260 nm detection) at t = 0 (top), 2 h (center) and 24 h (bottom).

3.- Photoactivation studies

Photoactivation studies with green light were performed at 37 ^{o}C in a custom-built irradiation setup from Microbeam, which includes a cuvette, thermostated cuvette holder, and mounted high-power LED (505±35 nm, 100 mW cm^{-2}). In a typical experiment, the cuvette containing 1.5 mL of a solution of the compounds in Milli-Q H_2O (20 μM) was placed in front of the light source and irradiated for the indicated times while constantly stirred. The light source of a fluorimeter was used in photoactivation studies with blue light (150 W lamp, slits 5 nm (1.25 mm)). At each time point, samples were taken and analyzed by reversed-phase HPLC-ESI MS with a Jupiter Proteo C_{18} column (250×4.6 mm, 90 Å, 4 μm, flow rate: 1 mL min^{-1}) by using linear gradients of 0.1% formic acid in H_2O (A) and 0.1% formic acid in ACN (B).

In NMR photoactivation experiments, the sample containing the compounds was dissolved in a 8:2 (v/v) mixture of DMSO-d_6 and D_2O and irradiated with green light (505±35 nm, 100 mW cm^{-2}) with continuous stirring at 37 ºC for the time indicated. After irradiation, the sample was transferred into a NMR tube and the ^{1}H NMR spectrum was recorded at 25 ^{o}C in a 400 MHz spectrometer.

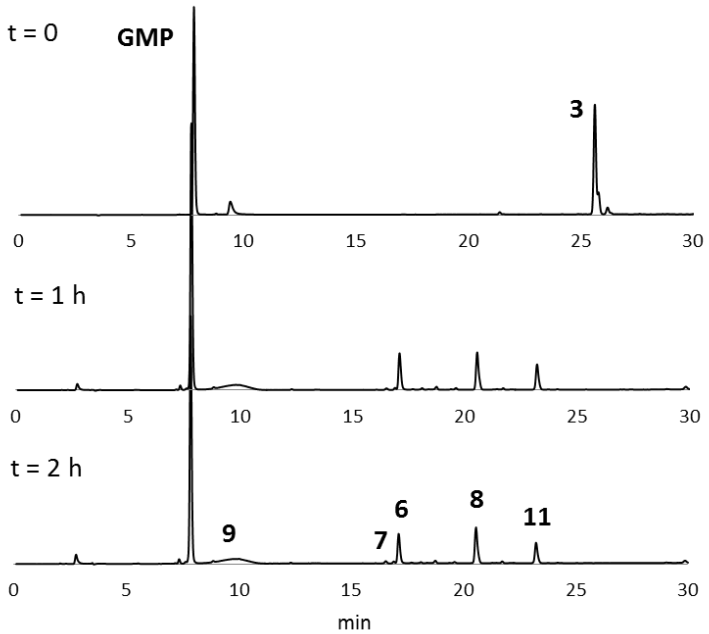

Figure S6. Reversed-phase HPLC-MS analysis (260 nm detection) of the photoactivation of Pt-folate conjugate **3** in the presence of 5'-GMP (2 mol equiv.) in H_2O with green light.

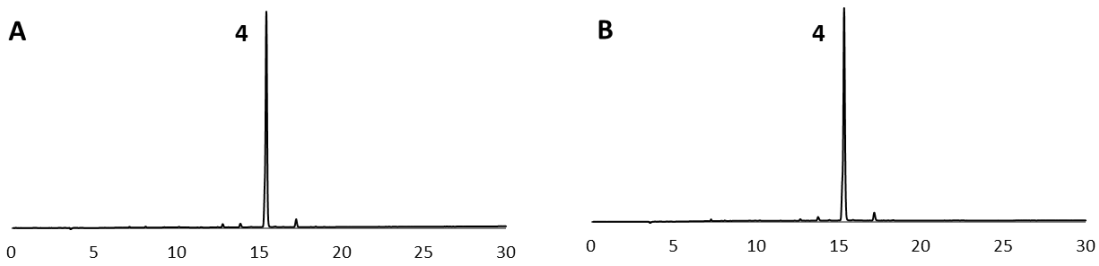

Figure S7. Reversed-phase HPLC-MS analysis (260 nm detection) for the irradiation of folate derivative **4** with blue (A) or green (B) light.

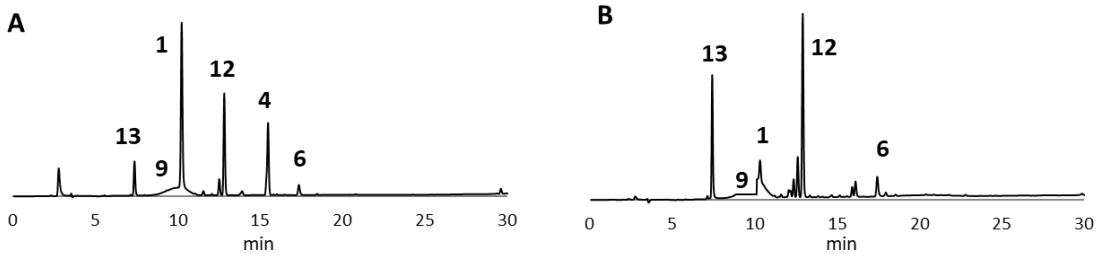

Figure S8. Reversed-phase HPLC-MS analysis (260 nm detection) for the irradiation of an equimolar mixture of complex **1** and folate derivative **4** with blue (A) or green (B) light. For assignment of peaks see Scheme 2.

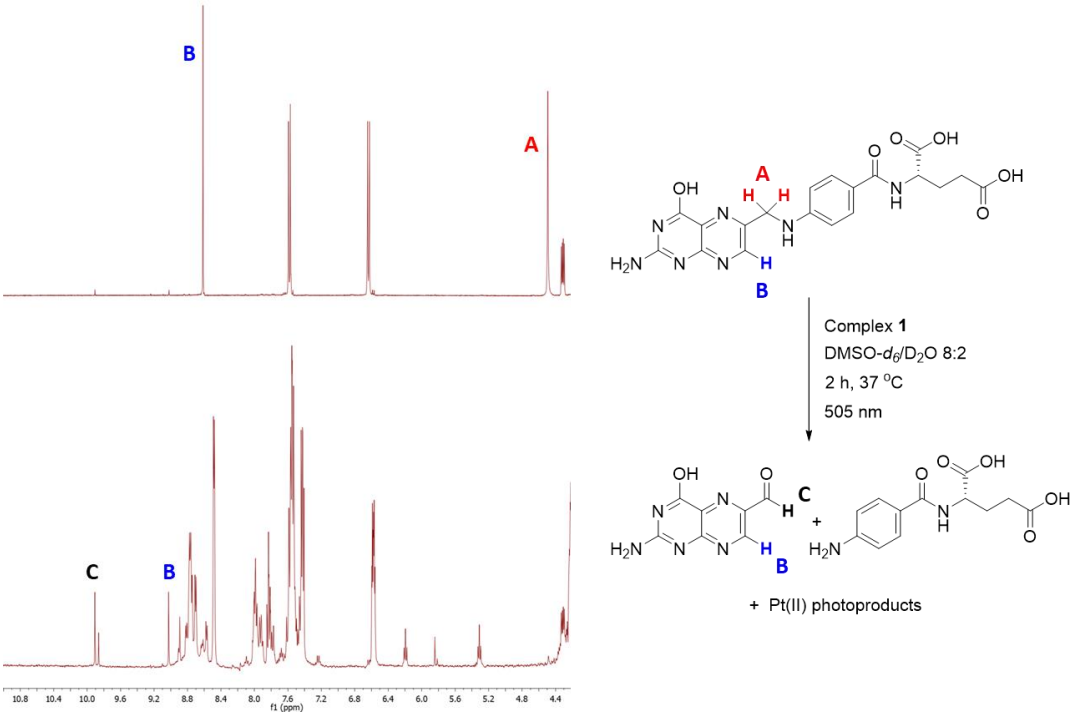

Figure S9. ^{1}H NMR spectra of folic acid alone (top) and after irradiation with green light for 2 h at 37 ^{o}C in the presence of complex **1** (bottom) in DMSO-d_6/D_2O 8:2 (v/v). The region between 4 and 11 ppm is shown.

4.- Biological studies

Cell culture. Human lung adenocarcinoma A549 and breast cancer MCF7 cells were obtained from the European Collection of Animal Cell Culture (ECACC), Salisbury, UK. All cell lines used in this work were grown in Roswell Park Memorial Institute medium (RPMI-1640), which was supplemented with 10% v/v of foetal calf serum (FCS) and 1% v/v penicillin/streptomycin. The adherent monolayers of cells were grown at 310 K in a humidified atmosphere containing 5% CO_2 and passaged regularly at *ca*. 80% confluence.

Photo-dark cytotoxicity. Approximate 1.5×10^4 cells were seeded per well in 96-well plates. The cells were pre-incubated in drug-free medium with phenol red at 310 K for 24 h. Complexes were dissolved first in DMSO and then diluted in DMEM without phenol red and folate to make the stock solution of the drug, which were further diluted using phenol-red and folate-free DMEM until working concentrations were achieved. The maximum DMSO concentration was < 0.5% v/v in these solutions. Cells were exposed to the drugs at various concentrations for 1 h. Then one plate was irradiated for 1 h using blue light (4.8 mW cm^{-2} per LED at 465 nm), while the dark plate was kept in the incubator. After irradiation, supernatants of both plates were removed by suction and the cells were washed with phosphate-buffered saline (PBS). Photocytotoxicity was determined after another 24 h recovery at 310 K in drug-free phenol red-containing RPMI-1640 medium by comparison to untreated controls which were only exposed to vehicle. Untreated controls were also compared between the irradiated and the non-irradiated plates to ensure that the differences in cell survival were not statistically relevant, hence guaranteeing that the differences in cell viability observed were not due to the light source. The SRB assay was used to determine cell viability.[2] Absorbance measurements of the solubilised dye (on a Promega microplate reader) allowed the determination of viable treated cells compared to untreated controls. IC_{50} values (concentrations which caused 50% of cell death) were determined as the average of triplicates and their standard deviations were calculated. Stock concentrations for all metal complexes used in these biological assays were adjusted/verified after ICP-OES metal quantification.

Platinum accumulation in cancer cells in dark. For Pt cellular accumulation studies, *ca*. 3.5×10^6 MCF7 cells were seeded in 100 mm Petri dishes and allowed for 24 h to attach. Then the plates were exposed to complexes at 10 μM in the presence or absence of 100 mol. equiv. of folate. Additional plates were incubated with medium alone as a negative control. After 1 h of incubation in the dark at 277 K or 310 K, the cells were rinsed three times with cold PBS and harvested by trypsinisation. The number of cells in each sample was counted manually using a haemocytometer. Then the cells were centrifuged to obtain the whole cell pellet for ICP-MS analysis. All experiments were conducted in triplicate.

5.- References

1. S. Dhar, Z. Liu, J. Thomale, H. Dai, S. J. Lippard. *J. Am. Chem. Soc*. **2008**, *130*, 11467-11476.

2. V. Vichai, K. Kirtikara, *Nat. Protoc*., **2006**, *1*, 1112-1116.