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SUPPORTING INFORMATION

Iodinated Cyanine Dyes: A New Class of Sensitisers for use in NIR Activated Photodynamic Therapy (PDT)

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S1.0 Experimental Section

S1.1 Materials and Methods: Chemicals were purchased from commercial sources at the highest possible purity and used as received. Singlet oxygen Sensor Green (SOSG) was purchased from Invitrogen (UK). UV-Vis spectra were recorded with a Varian Cary spectrometer, using quartz cells with a path a length of 1 cm. NMR spectra were obtained on Varian 500 MHz instrument at 25.0 ± 1 ^{o}C and processed using Bruker software. Mass spectra were obtained using a Finnegan LCQ-MS instrument. Emission spectra were recorded using a Varian Cary Eclipse spectrometer in aerated solutions, slits = 5 nm.

*S1.2 Synthesis of (4-iodophenyl)hydrazine and (3,5-diiodophenyl)hydrazine (**2a, 2b**):* A solution containing 4-iodoaniline (**1a**, 20g, 91.3 mmol) and hydrochloric acid (5.5 M, 15 mL) was cooled to -10^{o}C and $NaNO_2$ (12.6g, 182.6 mmol) in 45 mL of water was added drop wise with continuous stirring. The suspension was allowed to stir for 30 min and then an ice cold solution of $SnCl_2.2H_2O$ (67.99g, 301.3 mmol) in 40 mL of concentrated HCl was added drop wise keeping the temperature at -10^{o}C. The reaction mixture was stirred at that temperature for 1.5 hr and at then for a further 18 h at 5^{o}C. The light brown precipitate obtained was filtered and washed three times with water. This solid was then stirred with a saturated NaOH solution

(100 mL) and extracted with ether (200 mL). The ether layer was washed with a saturated aqueous solution of NaOH (50 mL X 2), $Na_2S_2O_3$ (50 mL X 2) and finally with water (100 mL). After drying over anhydrous $MgSO_4$, the ether layer was evaporated to dryness to afford **2a** (17.94 g, 84%) as brown powder. mp 104-106^oC, ^1H NMR ($CDCl_3$): 7.48 (d, *J*=8.0 Hz, 2H, Ar-CH), 6.62 (d, *J* = 8.0 Hz, 2H, Ar-CH), 5.18(brs, 1H, NH), 3.55 (brs, 2H, NH_2). ESMS (M+H) found = 235.0, calculated for $C_6H_7IN_2$ = 234.04.

2b was obtained by the same procedure using 5g (14.5 mmol) of **1b**.[1] The product **2b** was isolated as fine brown needles (Yield= 72%). ^1H NMR ($CDCl_3$) δ = 8.43 (d, *J* = 1.4 Hz, 2H, Ar-H X2), 8.29 (s, 1H, Ar-CH); ^{13}C NMR (CDCl3) δ: 94.1, 131.7, 148.4, 151.0; ESMS [M+H$^+$]: calculated for $C_6H_3I_2NO_2Na$ = 397.8, found = 398.9 m/z.

*S1.3 Synthesis of 5-iodo-2,3,3-trimethyl-3H-indole and 4,6-diiodo-2,3,3-trimethyl-3H-indole (**3a**, **3b**):* To a refluxing solution of (4-iodophenyl) hydrazine (**2a**, 12.68g, 54.1 mmol) in glacial acetic acid (100 mL), was added 3-methyl-2-butanone (8g, 92.8 mmol) in a dropwise manner. After a further 20 hr reflux, excess acetic acid was removed under reduced pressure and the residue was dissolved in ether (100 mL). The resulting insoluble precipitate was removed by filtration and the ether solution washed with a saturated aqueous solution of NaOH (50mL X 2), $Na_2S_2O_3$ (50 mL X 2) and finally with water (100 mL). The organic layer was dried using anhydrous Na_2SO_4, filtered and the ether removed under reduced pressure to afford **3a** (10.5g 68%) as red gummy liquid. ^1H NMR ($CDCl_3$): δ 7.60 (dd, *J* = 4.5, 8.0 Hz, 2H, Ar-CH), 7.28 (d, *J* = 8.0 Hz, 1H, Ar-CH), 2.25 (s, 3H, CH_3), 1.20 (s, 6H, CH_3 X 2). ^{13}C NMR ($CDCl_3$): 153.4 (C), 148.1 (C), 139.3 (C), 136.6 (CH), 130.6 (CH), 121.8 (CH), 89.9(C), 54.0 (C), 23.0 (CH_3), 22.9 (CH_3), 15.3 (CH_3). ESMS (M+H) found = 286.1, calculated for $C_{11}H_{12}IN$ = 285.12. **3b** was obtained by literature procedure[2] and was isolated as red gummy liquid. ^1H NMR ($CDCl_3$): 7.88 (s, 1H, Ar-CH), 7.76 (s, 1H, Ar-CH), 2.21 (s, 3H, -CH_3), 1.33 (s, 6H, -CH_3 X 2). ^{13}C NMR ($CDCl_3$): 190.7 (C), 156.8(C), 145.6(C), 142.6(CH), 129.1(CH), 93.1(C), 90.3(C), 56.8(C), 19.3(CH_3), 15.7(CH_3). ESMS (M+H) found = 434.4, calculated for $C_{11}H_{11}I_2NNa$ = 433.90.

*S1.4 Synthesis of 5-iodo-2,3,3-trimethyl-1-(4-sulfobutyl)-3H-indol-1-ium and 4,6-diiodo-2,3,3-trimethyl-1-(4-sulfobutyl)-3H-indol-1-ium (**4a**, **4b**):* Toluene (70 mL) was added to a mixture of 5-iodo-2,3,3-trimethyl-3*H*-indole (**3a**, 12g, 42.1 mmol) and 1,4-butane sultone (8.6g, 63.1 mmol) in a round bottomed flask and the mixture heated under reflux for 18 hrs. After cooling to room temperature the resulting brown crystals were filtered and washed with acetone (3 X 10 mL). The solid was recrystallized from a solution of MeOH and diethyl ether (30%v/v). The crystals were collected and dried *in vacuo* to yield **4a (**8g, 45%). ^{1}H NMR (dmso-d_6): δ 8.27(s, 1H, Ar-CH), 7.95 (s,1H, Ar-CH), 7.82(s, 1H, Ar-CH), 4.42 (brs, 2H, CH_2), 2.79 (s, 3H, CH_3), 2.47 (brs, 2H, CH_2), 1.90 (brs, 2H, CH_2), 1.69 (brs, 2H,CH_2), 1.49 (s, 6H, CH_3 X 2). ^{13}C NMR (DMSO-d_6):176.2, 148.4, 139.9, 136.7, 132.5, 126.8, 96.8, 49.8, 46.8, 42.6, 26.8, 25.6, 10.5. ESMS (M+H) found = 422.10, calculated for $C_{15}H_{21}INO_3S^+$ = 422.30.

4b was synthesized following a similar protocol from **3b** and isolated as reddish brown crystals (Yield = 56%). mp 107-109^{o}C. ^{1}H NMR (MeOH-d_4): δ 8.42 (s, 1H, Ar-CH), 8.36 (s, 1H, Ar-CH), 4.51-4.48 (m, 2H, CH_2), 2.88-2.85 (m, 2H, CH_2), 2.09-2.00 (m, 2H, CH_2), 1.99-1.82 (m, 2H, CH_2), 1.73 (s, 6H, CH_3 X 2), 1.16 (s, 3H, CH_3). ESMS [M-H$^+$]: calculated for $C_{15}H_{20}I_2NO_3S^+$ = 547.9, found = 546.1 m/z.

*S1.5 Synthesis of 2-((E)-2-((E)-2-chloro-3-((E)-2-(5-iodo-3,3-dimethyl-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-5-iodo-3,3-dimethyl-1-(4-sulfobutyl)-3H-indol-1-ium (**6a**) and 2-((E)-2-((E)-2-chloro-3-((E)-2-(4,6-diiodo-3,3-dimethyl-1-(4-sulfobutyl)indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-4,6-diiodo-3,3-dimethyl-1-(4-sulfobutyl)-3H-indol-1-ium (**6b**):* A solution of **4a** (0.2g, 0.47mmol), (*E*)-*N*-(((*E*)-2-chloro-3-((phenylamino) methylene) cyclohex-1-en-1-yl) methylene)benzenaminium[3] (**5**, 0.153g, 0.47 mmol) and anhydrous sodium acetate (0.077g, 0.93 mmol) in absolute EtOH (10 mL) under a N_2 atmosphere was heated under reflux for 4 hr. Excess EtOH was removed under reduced pressure and the residue purified by column chromatography (silica 60-120 mesh) using 25% MeOH-$CHCl_3$ mixture as elutant. The product **6a** was isolated as greenish powder (0.152g, 33%). mp 131-133^{o}C. ^{1}H NMR (MeOH-d_4): δ 8.26 (d, *J* =7.8 Hz, 1H, Ar-CH), 8.03-7.98 (m, 2H,

Ar-CH), 7.68-7.63 (m, 2H, Ar-CH), 7.63-7.49 (m, 1H, Ar-CH), 6.39-6.36 (m, 2H, CH X 2), 4.34-4.33 (m, 2H, CH X 2), 3.33-3.34 (m, 4H, CH_2 X 2), 2.92-2.90 (m, 2H, CH_2), 2.89-2.80 (m, 2H, CH_2), 2.08-1.96 (m, 26H, CH_2 X 7, CH_3 X 4). ^{13}C NMR (DMSO-d_6): 174.7, 173.9, 150.1, 149.6, 148.0, 146.7, 145.9, 130.8, 134.8, 132.6, 130.1, 129.8, 128.3, 126.4, 124.7, 120.7, 116.1, 114.9, 104.6, 102.8, 98.6, 62.1, 60.1, 50.4, 29.1, 48.7, 30.5, 28.4, 28.5, 26.3, 26.2, 24.6. ESMS ($M-H^+$) found = 977.2, calculated for $C_{38}H_{46}ClI_2N_2O_6S_2^+$ = 979.06.

6b was synthesized following a similar protocol from **4b**. The product was isolated as brown powder (Yield = 41%). mp 111-114^{o}C. ^{1}H NMR (MeOH-d_4): δ 8.59 (s, 2H, Ar-CH X 2), 8.29 (s, 2H, Ar-CH X 2), 6.77-6.75 (m, 2H, CH X 2), 5.30 (brs, 2H, CH X 2), 4.82-4.72 (m, 4H, CH_2 X 2), 3.39 (brs, 4H, CH_2 X 2), 2.60-2.47 (m, 14H, CH_2 X 7), 2.23 (s, 12H, CH_3 X 4). ^{13}C NMR (DMSO-d_6): 170.2, 169.9, 158.9, 150.1, 149.7, 148.6, 146.8, 144.9, 140.8, 139.3, 134.2, 132.1, 126.7, 124.3, 104.0, 100.4, 96.7, 96.2, 94.5, 64.1, 59.5, 50.5, 48.7, 48.1, 30.3, 28.7, 28.2, 26.3, 26.1, 24.3. ESMS [$M-H^+$]: calculated for $C_{38}H_{44}Cl_4N_2O_6S_2Na^+$ = 1253.85, found 1252.81 m/z.

*S1.6 Determination of singlet oxygen generation of **ICG, 6a and 6b**:* PBS (3 mL, pH = 7.4 ± 0.1) solutions containing *ICG, **6a** and **6b*** (5 μM) and singlet oxygen sensor green (2.5 μM) were prepared. The solutions were irradiated with NIR light (780 nm, 100 mW) for 10 min. The fluorescence intensity of SOSG (λ_{EX} = 505 nm) was recorded at 525 nm at the beginning and end of the experiment.

*S1.7 PDT treatment of BxPC-3 and MiaPaCa-2 cells using ICG, **6a**, **6b** and 5-FU:* BxPC-3 and MiaPAca-2 cells were cultured in DMEM, supplemented with foetal bovine serum (10 % v/v) and penicillin (200 μg mL^{-1}). The cells were seeded in 384 well plates at a density of 2 x 10^3 cells per well, incubated for 24h at 37°C in a humidified CO_2 (5%) atmosphere and spiked with PBS solutions (pH = 7.4 ± 0.1) containing ICG, **6a** or **6b** prepared to a final concentration of 6.25, 12.5, 25 and 50 μM. The cells were incubated in the dark for a further 3 h, the medium was removed and each well was washed twice with PBS. Fresh PBS (100μL) was added to

each well and selected wells treated with NIR light (780nm, 100mW) for 1 min. After light treatment the PBS was removed, replaced with fresh medium (100μL) and cells were allowed to incubate in the dark for a further 24 hrs at 37°C in a humidified CO_2 (5%) atmosphere. Cell viability was then determined using a MTT assay.[4] For the determination of 5-FU toxicity, the same cell lines were incubated with 5-FU at 50, 100 and 250 μM for 24 h and cell viability then determined using the MTT assay. Cell viability was expressed as a percentage of a control, untreated cell population.

S1.10 In vivo PDT and Imaging experiments: All animals employed in this study were treated humanely and in accordance with licenced procedures under the UK Animals (Scientific Procedures) Act 1986 and approved by the Animal Welfare Ethical Review Board (AWERB) at Ulster University. BxPC3-Luc xenograft tumours were established on the dorsum of SCID mice by subcutaneous injecting 2 x 10^6 cells suspended in 100 μl of matrigel with a 25 gauge needle. Once the tumour became palpable dimensions were measured using calipers. The geometric mean diameter (GMD) was calculated as: GMD = $\sqrt[3]{}$ length x breadth x height. Treatment commenced when tumour volumes reached 200-250mm^3 (Day 0). Mice were anaesthetised by i.p. injection using a 100μl mixture of 2:1:1 PBS, Hypnorm and Hypnovel. Groups were untreated or treated with **6a** (2.5 mg/kg in PBS injected intratumourally) followed by exposure to light at 780nm for 3 x 3 min. periods interspaced by 1min on Day 0 and Day 8. Tumour volumes were recorded from pre-treatment up until Day 11.

S1.11. In vivo fluorescence imaging: For the tumour localisation study, **6a** (6.0 mg/kg in PBS provide injection volume) was administered by tail vein injection into a BxPC3-Luc xenograft (was the animal anaesthetised and compared against an untreated control. Whole body fluorescence imaging was performed using a Xenogen IVIS imaging system using bioluminescent and fluorescent modes after 30 second exposure time. In the fluorescence imaging mode the ICG excitation and emission filter set was employed. Images were captured

and analysed use the Living Image software package. Where surgically-excised tumours were imaged, animals were sacrificed and tumours were surgically excised at 18h post injection.

Additional Figures & Diagrams

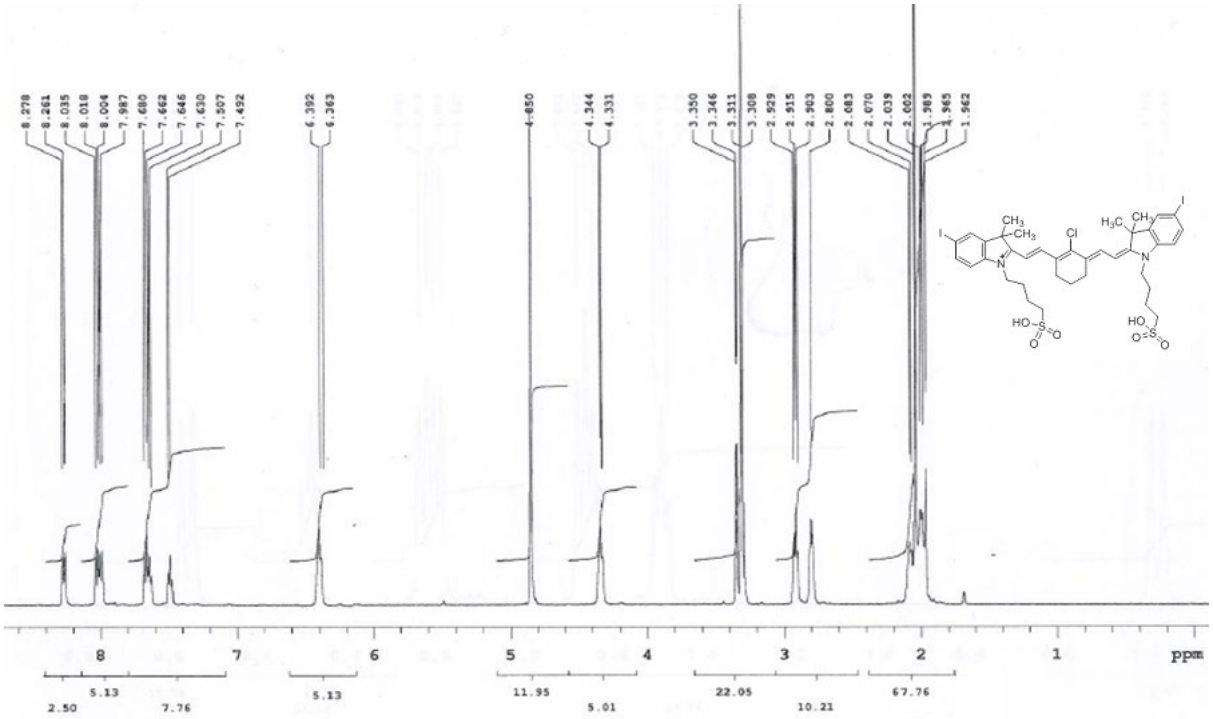

Figure S1 ^{1}H NMR spectrum of **6a** recorded in MeOH-d_4.

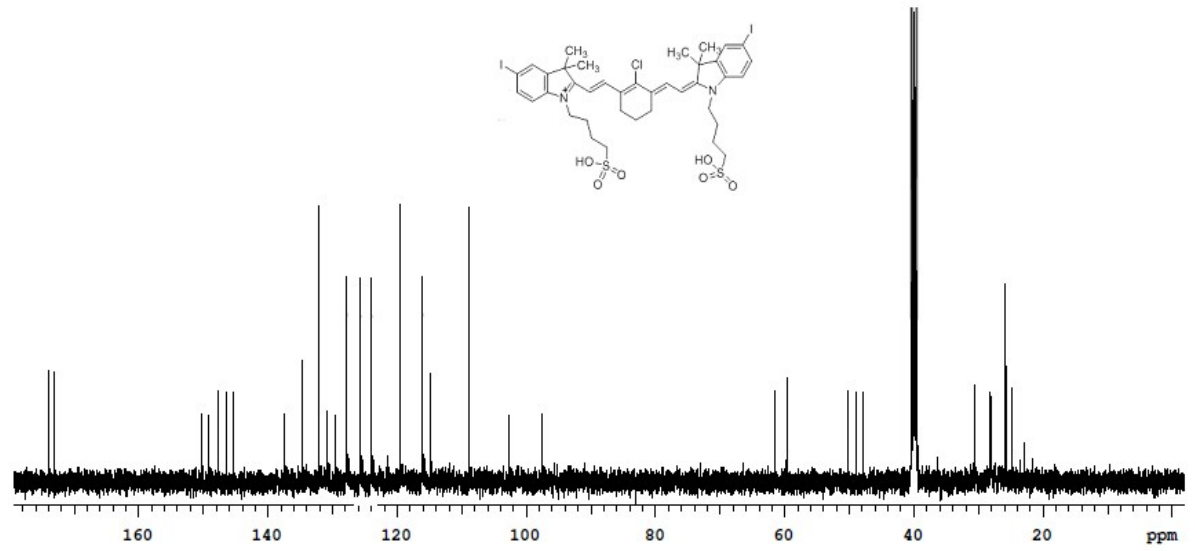

Figure S2 ^{13}C NMR spectrum of **6a** recorded in MeOH-d_4

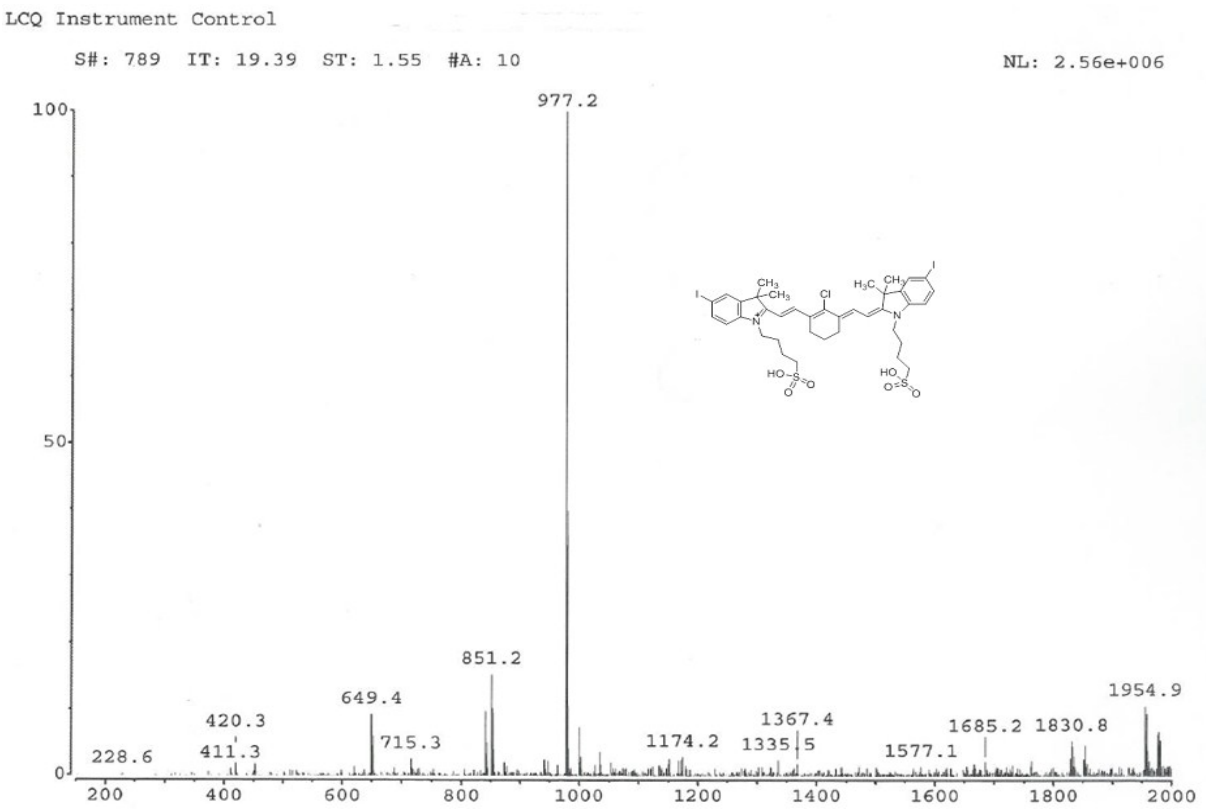

Figure S3 Positive electrospray mass spectrum of **6a.**

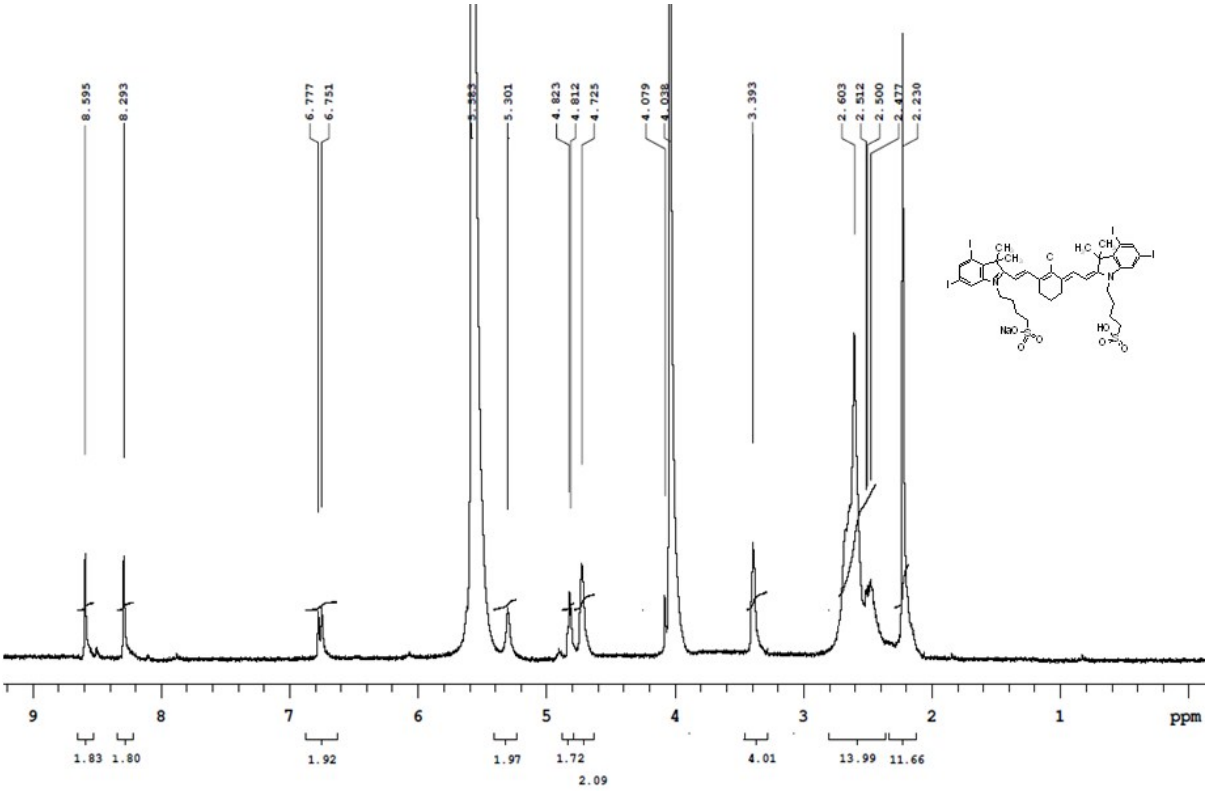

Figure S4 ^{1}H NMR spectrum of **6b** recorded in MeOH-d_4.

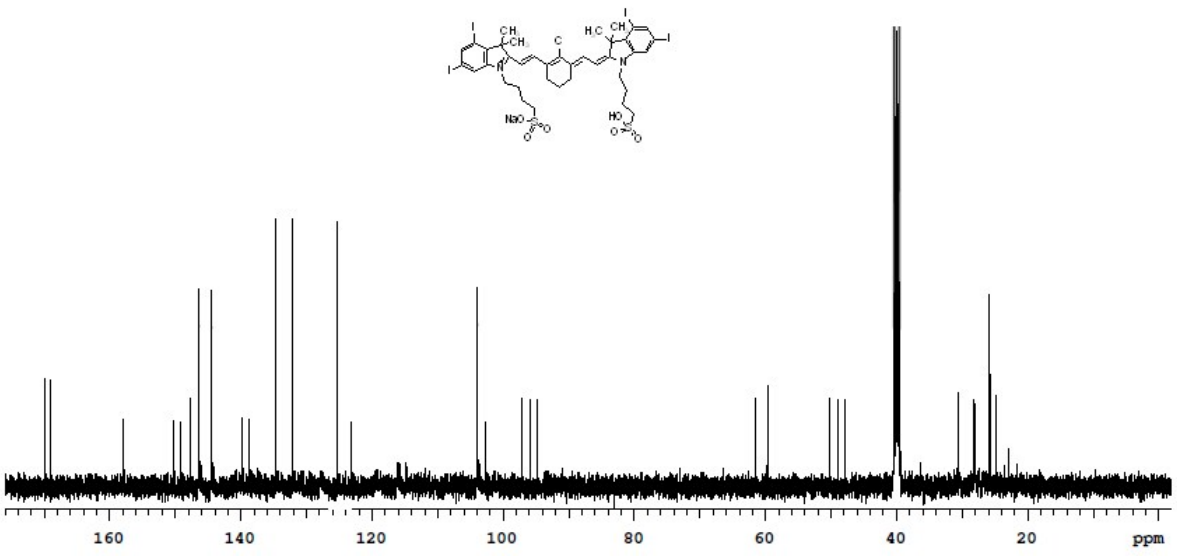

Figure S5 ^{13}C NMR spectrum of **6b** recorded in MeOH-d_4.

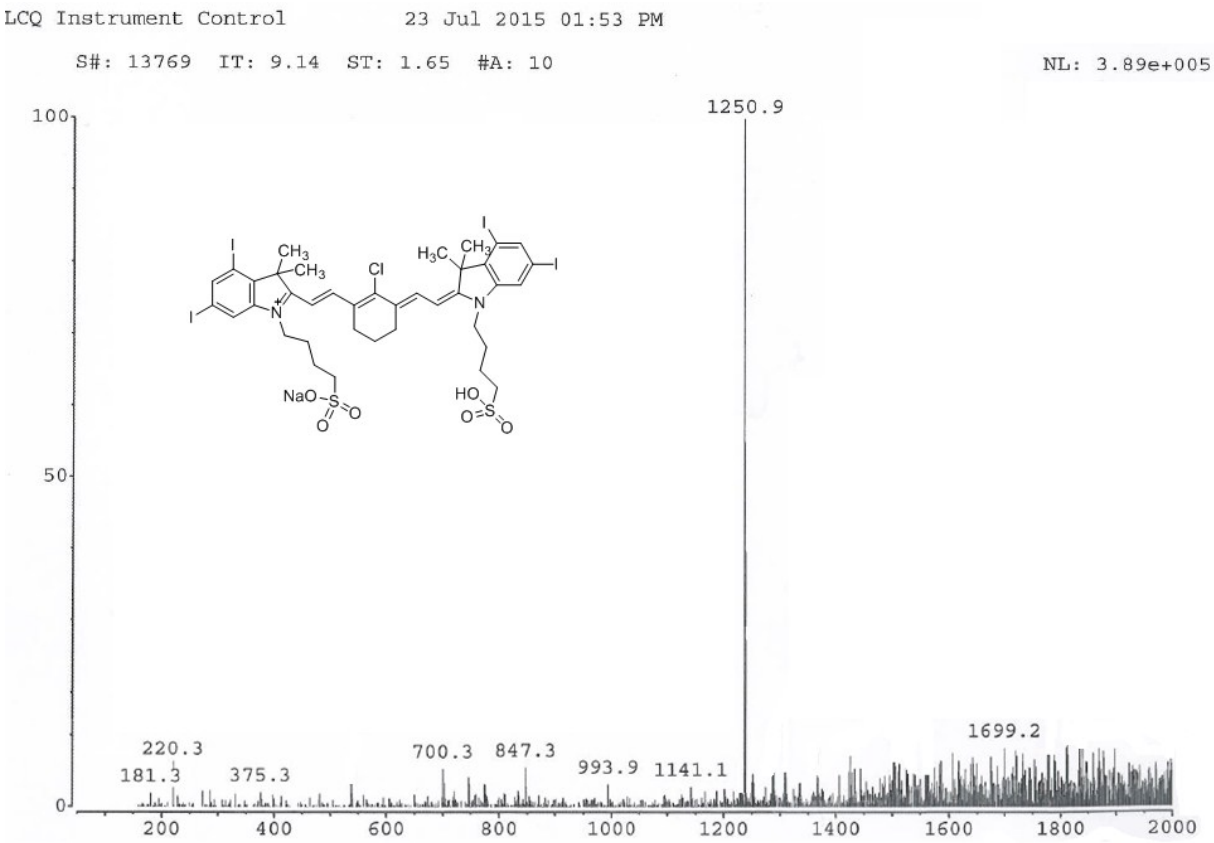

Fig S6 Negative electrospray mass spectrum of **6b**

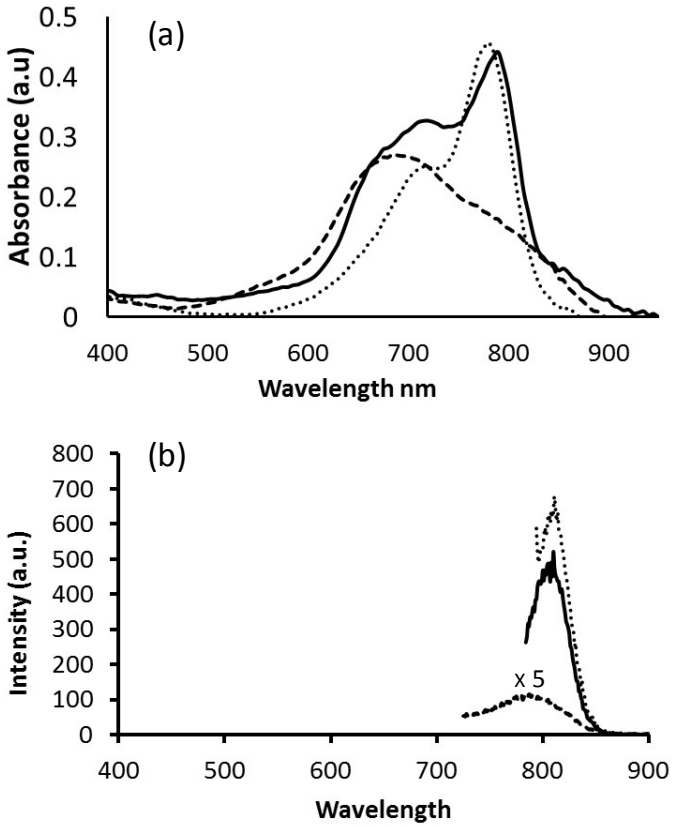

Fig S7 (a) UV-Vis spectra and (b) Fluorescence spectra of ICG (……), **6a** (solid line) and **6b** (-----). Solvent = H_2O, [ICG] = 3.9 μM [**6a**] = [**6b**] = 10 μM.

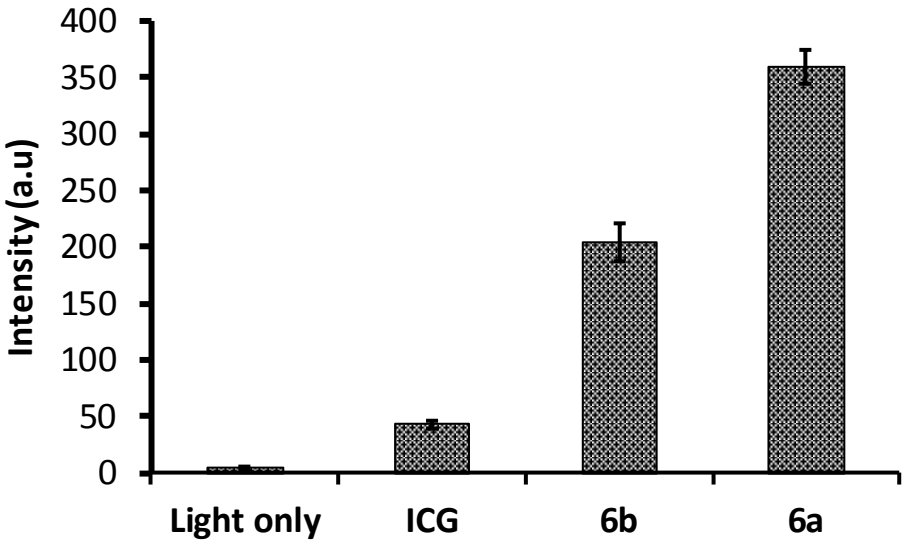

Fig S8 Plot of increase in SOSG intensity at 410 nm for solutions of ICG, **6a** and **6b** recorded 10 min after treatment with a 780 nm laser. The effect of light treatment alone on the fluorescence of SOSG is also shown for completeness.

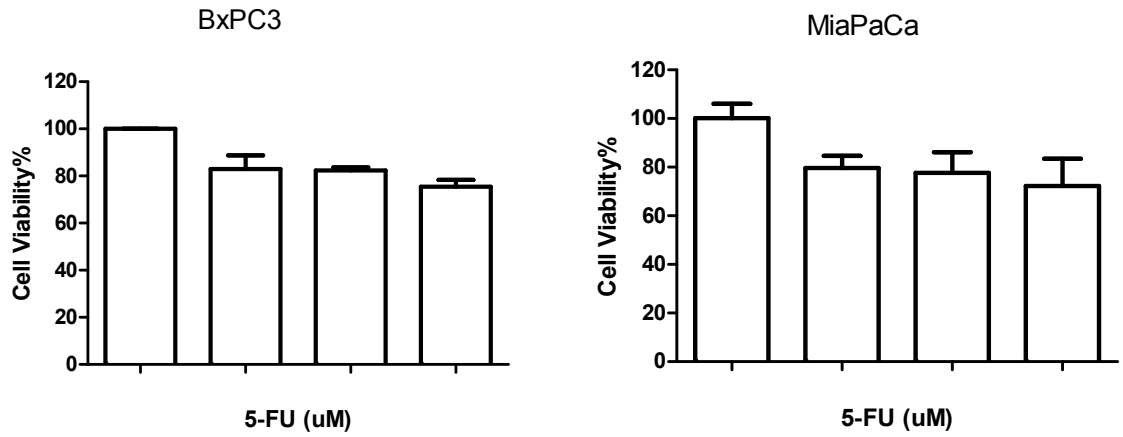

Fig S9 Plot of cell viability against concentration for the antimetabolite drug 5-fluoruracil (5-FU) determined 24 h after incubation in BxPC-3 and MiaPaCa-2 cells.

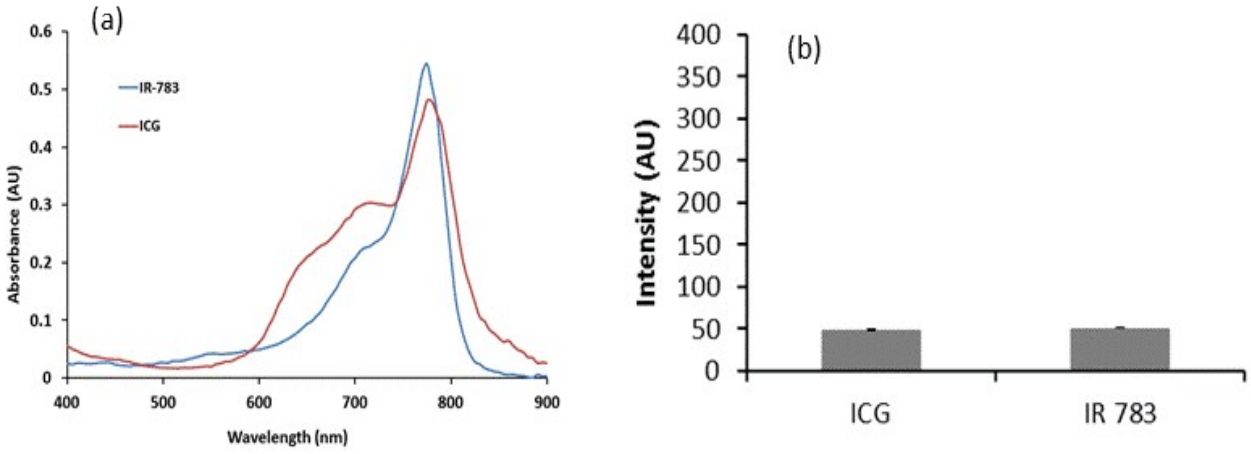

Figure S10 (a) UV-Vis spectra of ICG and IR 783 (b) Plot of increase in SOSG intensity at 410 nm for solutions of ICG and IR-783 10 min after treatment with a 780 nm laser. [ICG] = [IR 783] = 5 μM.

References

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