Switching-on Prodrugs using Radiotherapy

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1. General information

Anhydrous solvents were purchased from Sigma Aldrich. PeproTech. Imidazole-1-sulfonylazide hydrochloride was purchased from Fluorochem.Hoechst 33342 and Click-iT™ EdU Alexa Fluor™ 488 were purchased from ThermoFisher. Celltiter Glo® was purchased

from Promega. μ-Dish and 8-well chambers were obtained from Ibidi. Corning® HTSTranswell® 96 well plates (8 μm pore) was purchased from Sigma Aldrich. All other chemicals were purchased from Sigma Aldrich and used without further purification.

X-ray source: A linear accelerator (Clinac iX from Varian Medical Systems) generated X-rays of nominal energy 6MV (with a Bremsstrahlung distribution of 2 – 6 MeV) were used, with a dose rate of 600 cGy per minute with samples treated at a depth of 1015 mm from the tungsten target with a build-up of 15 mm of solid water. The linear accelerator used was a Varian Medical Systems VitalBeam medical linear accelerator.

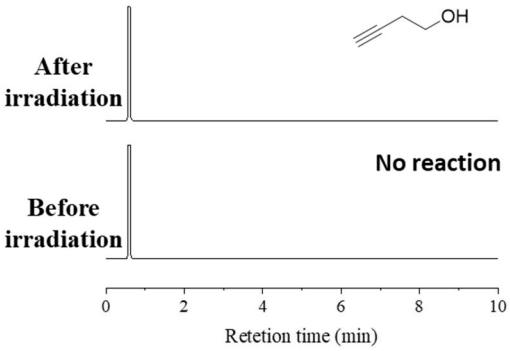
^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVA500 spectrometer (500 and 125 MHz, respectively) at 298 K in deuterated solvents. Chemical shifts for proton and carbon spectra are reported on the δ scale in ppm. All coupling constants (*J* values) were measured in Hz. High Resolution MS analysis were performed on a Bruker microTOF focus II msass spectrometer. Reverse phase analytical HPLC (RP-HPLC) were performed using an Agilent 1100 ChemStation using ChemStation B.03.017 software, with a Kinetex 5u XB-C18 (50 × 4 × 60 mm) column, with a flow rate of 1 mL/min using an evaporative light scattering detector (ELSD) (Polymer Lab PL-ELS 1000) with simultaneous detection at 220, 254, 260, 282 and 495 nm. Method A: Elution with 5% MeOH in water to 95% MeOH in water over 6 min and 95% MeOH in water for 4 min (both with 0.1% formic acid); Method B: Elution with 5% MeCN in water to 95% MeCN in water over 6 min and 95% MeCN in water for 4 min (both with 0.1% formic acid). All solvents used were HPLC grade. The fluorescence spectra were recorded using a FluoroMax-3 Jobin Yvon Div fluorimeter using DataMax v.2.20 software. Raw data were processed using Grapgpad Prism (6.01), Origin (8.5.1) and Excel (15.25).

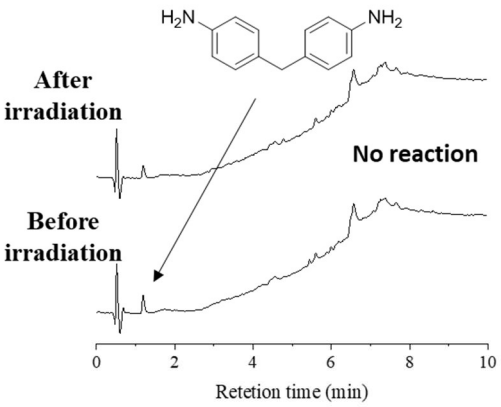
Prep-HPLC was performed with an Agilent Zorbax Eclipse® 5µm XDB-C18 column (250 × 10 mm, 5 µm), eluting with a gradient of H_2O/formic acid (0.1%) to ACN/formic acid (0.1%) over 25 min, with a flow rate of 2 mL/min.

Flow cytometry analysis was carried out on a Becton Dickinson (BD) FACSDiVa 2.2 software using the FlowJo software for data analysis. The absorbance and luminescence of 96-well plates were read on a BioTek HT Synergy multimode reader using the Gen 5.2.06.10 software. HeLa cells were monitored using a 20× objective (Leica fluorescence microscope using AxioVision 4.8.2.0 SP2 software) under brightfield and 488 nm excitation. Confocal images were taken on a Leica confocal microscope using LAS-AF-Lite_2.6.0 software for digital acquisition. Images were processed and analysed using ImageJ64 (1.51v).

Monitoring model reactions under X-ray irradiation

Selected model compounds (see chemical structures in Table 1 and Fig. S1 below) were dissolved in DMSO to give stock solutions of 100 mM and diluted in PBS (20 mL) to give a final concentration of 10 µM. The solutions were degassed by bubbling Ar for 30 min, analysed by HPLC and irradiated by the X-ray source. The reaction mixtures after irradiation were analysed by HPLC.


OH
After irradiation
No reaction
Before irradiation
0 2 4 6 8 10
Retetion time (min)


H2N
NH2
After irradiation
No reaction
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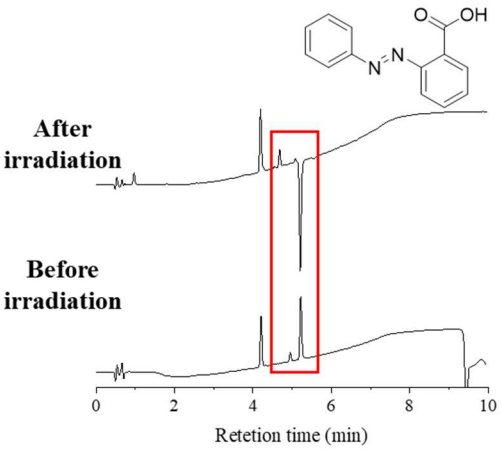
Retetion time (min)


O
OH
N
N
After irradiation
Before irradiation
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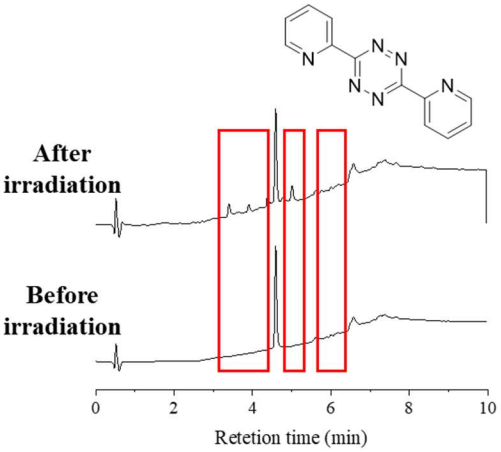
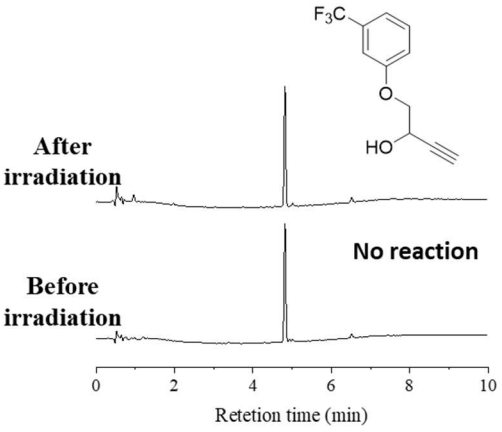
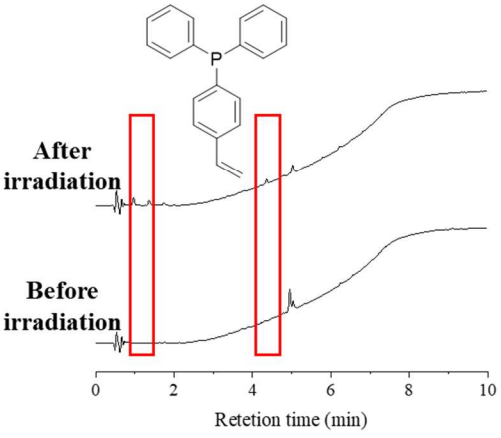
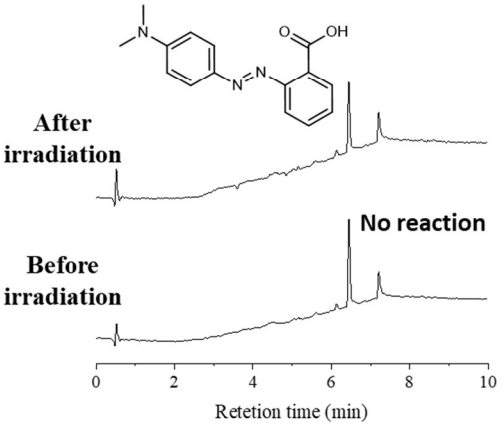
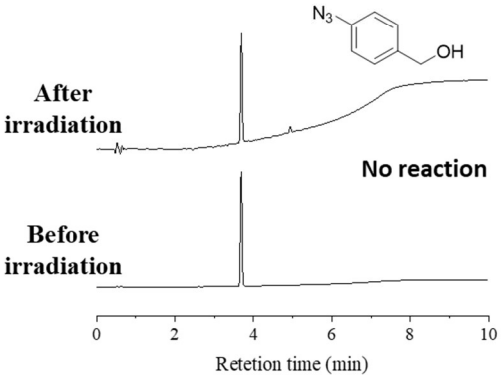
Retetion time (min)


N
N
N
N
N
After irradiation
Before irradiation
0 2 4 6 8 10
Retetion time (min)


F3C
O
HO
After irradiation
No reaction
Before irradiation
0 2 4 6 8 10
Retetion time (min)


P
After irradiation
Before irradiation
0 2 4 6 8 10
Retetion time (min)


N
O
OH
N
N
After irradiation
No reaction
Before irradiation
0 2 4 6 8 10
Retetion time (min)


N3
OH
After irradiation
No reaction
Before irradiation
0 2 4 6 8 10
Retetion time (min)

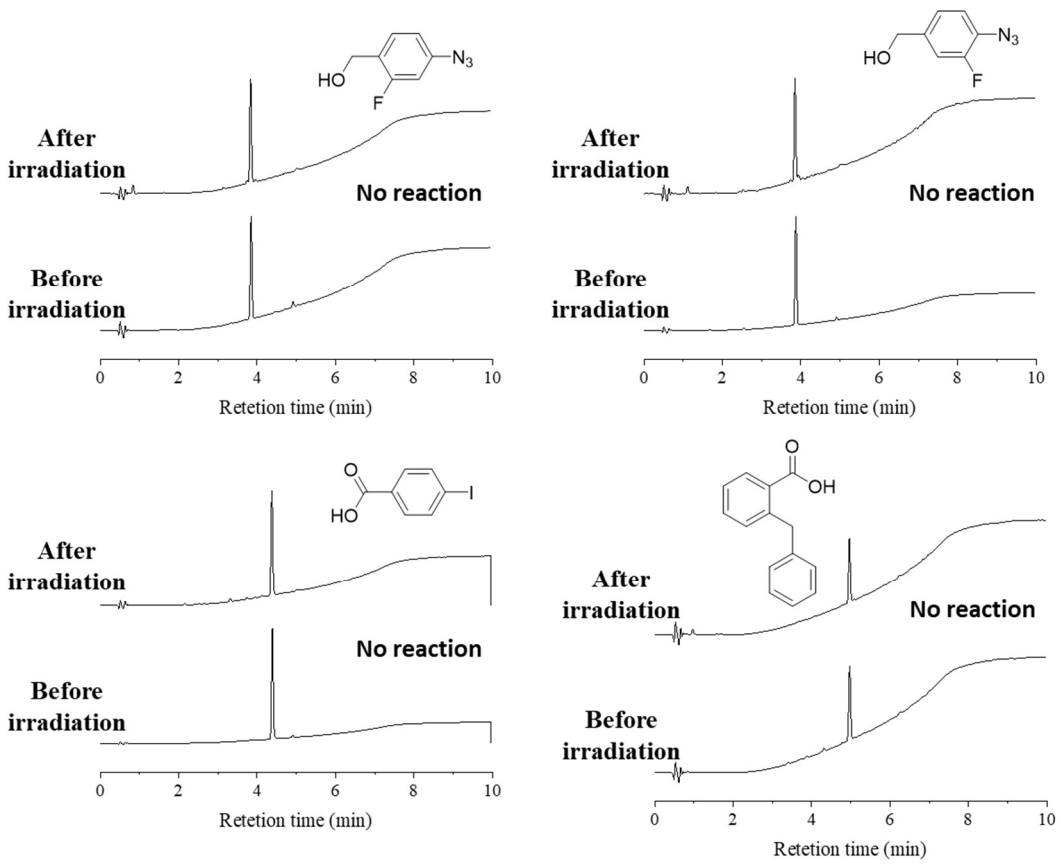
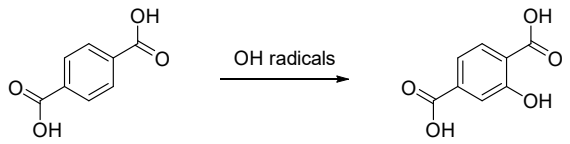


Fig. S1. HPLC traces of model reactions monitored (254 nm) before and after X-ray irradiation.

Calibration of OH radical concentrations by X-ray irradiation



Terephthalic acid (non-fluorescent) — 2-Hydroxyterephthalic acid (blue fluorescent)

The hydroxy radical concentration was quantified using terephthalic acid as an indicator as reported in the literature.[1] Briefly, fluorescence intensities of 2-hydroxyterephthalic acid (0.5, 1, 5, 10, 20 and 30 μM) in PBS were recorded using a fluorescence spectrometer (λ_{ex} = 320 nm) with the fluorescence intensities plotted versus terephthalic acid concentrations as a calibration curve. A stock solution of terephthalic acid (1 M in DMSO) was diluted in PBS (20

mL) to give a final concentration of 100 µM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation (6, 12, 24, 36, 48 and 60 Gy).

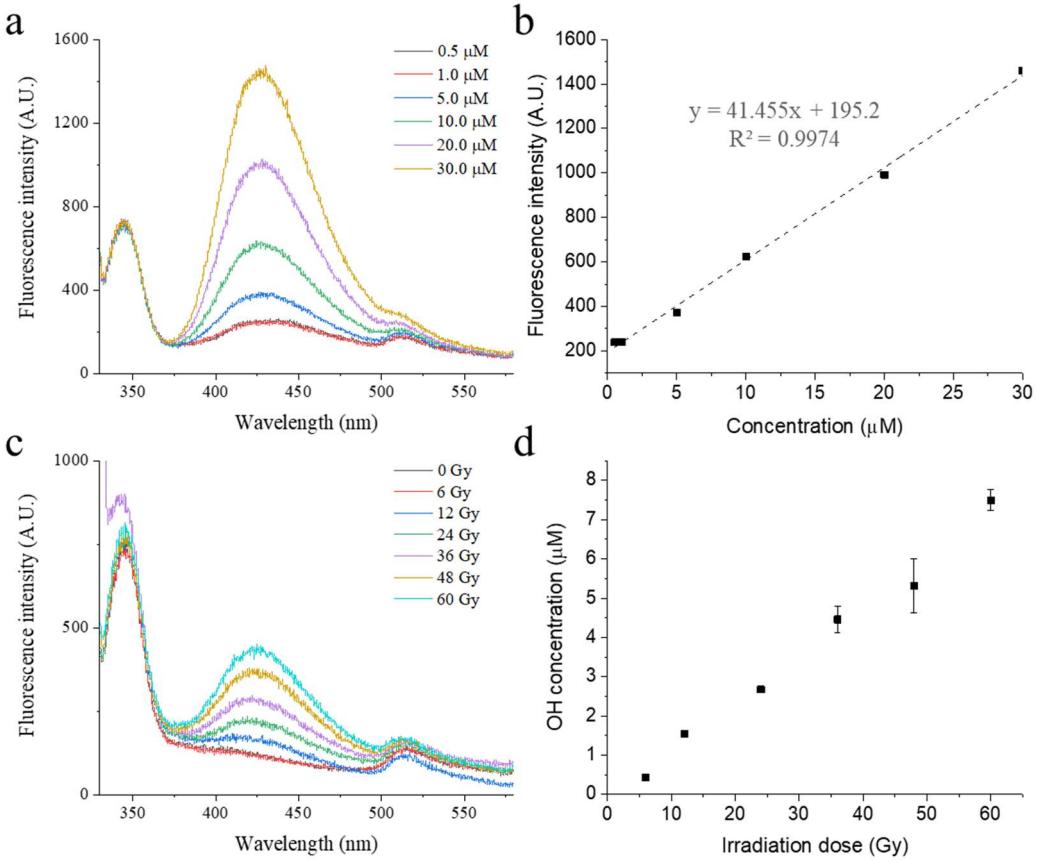
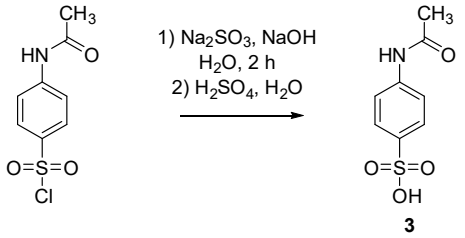


Fig. S2. (**a**) Fluorescence spectra of 2-hydroxyterephthalic acid in PBS (pH = 7.4) (λ_{ex} = 320 nm) at different concentrations and (**b**) the calibration curve of fluorescence intensities at 426 nm versus the concentration of 2-hydroxyterephthalic acid. The data are presented as mean ± SD (n = 3). (**c**) Fluorescence spectra of terephthalic acid (100 µM in PBS, pH = 7.4) irradiated at different doses and (**d**) the fluorescence intensities at 426 nm plotted versus irradiation doses. The data are presented as mean ± SD (n = 3). The experiments were repeated, independently, 3 times with similar results observed.

Synthesis of compound 3



Compound **3** was synthesised following a reported procedure giving a white solid in a 93% yield (4.8 g).[2]

^{1}H NMR (500 MHz, d-$CDCl_3$) δ (ppm) = 10.22 (s, 1H), 7.75 (d, J = 8.7 Hz, 2H), 7.58 (d, J = 8.7 Hz, 2H), 2.07 (s, 3H); ^{13}C NMR (125 MHz, d-$CDCl_3$) δ (ppm) = 169.38, 143.40, 141.52, 125.84, 119.93, 24.52; HRMS (EI) for $C_8H_9NO_4S$: *calcd*.: 215.0246; *found*: 215.0239.

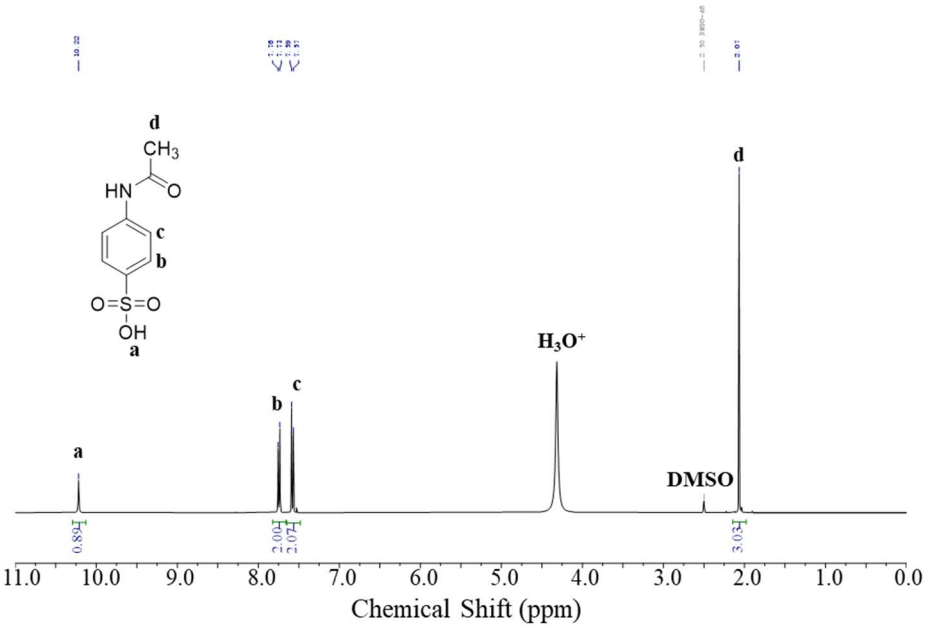
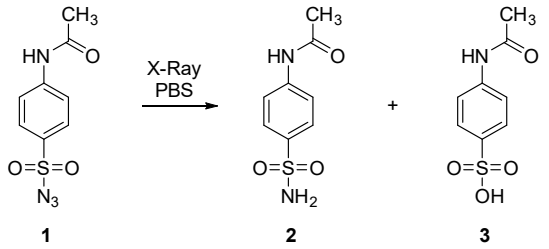


Fig. S3. ^{1}H NMR spectrum of compound **3** in d_6-DMSO.

Reaction of model compound 1 under X-ray irradiation



A stock solution of compound **1** (100 mM in DMSO) was diluted in PBS (20 mL) to give a final concentration of 20 μM. The solution was degassed by bubbling with Ar for 30 min before X-Ray irradiation. The reaction mixture was analysed by FTIR and following prep-HPLC, the isolated products were analysed by HRMS. The products co-eluted by HPLC [compared with synthesised compound **3** and commercially available compound **2** (CAS 121-61-9)]. (Compound **3** was not isolatable due to the limited quantity obtained).

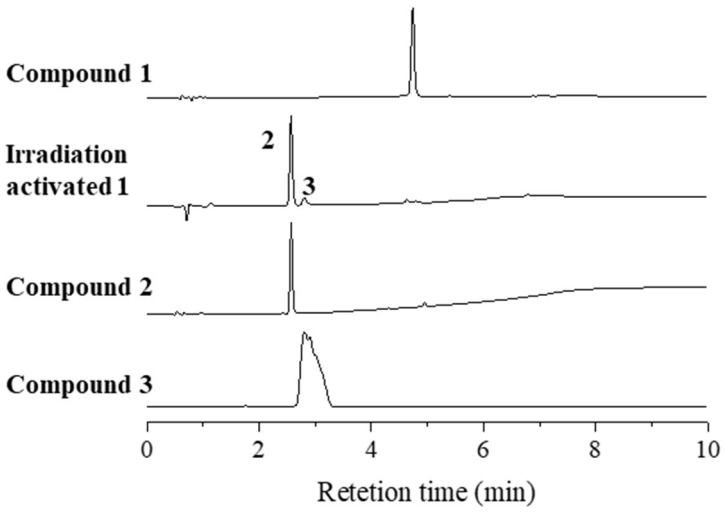


Fig. S4. HPLC traces of model compound **1**, irradiation activated **1**, compound **2** and compound **3**.

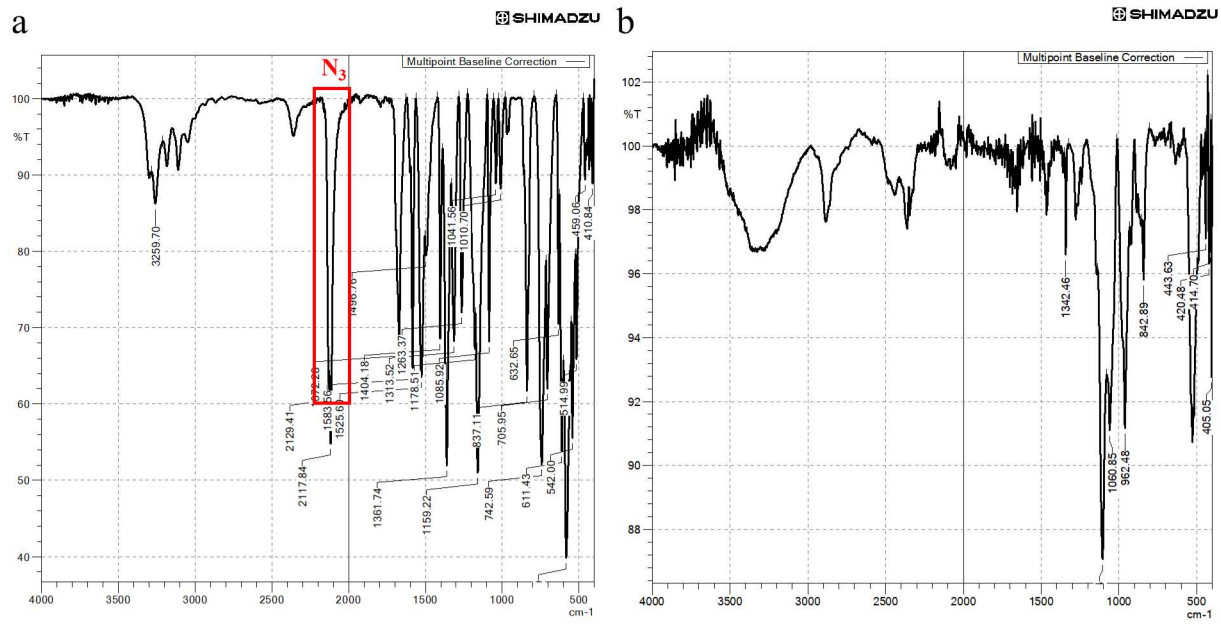


Fig. S5. FTIR spectra of (a) compound **1** and (b) irradiated **1** (60 Gy). The absence of the identical azide signal (2117.8 cm^{-1}) indicated the consumption of **1**.

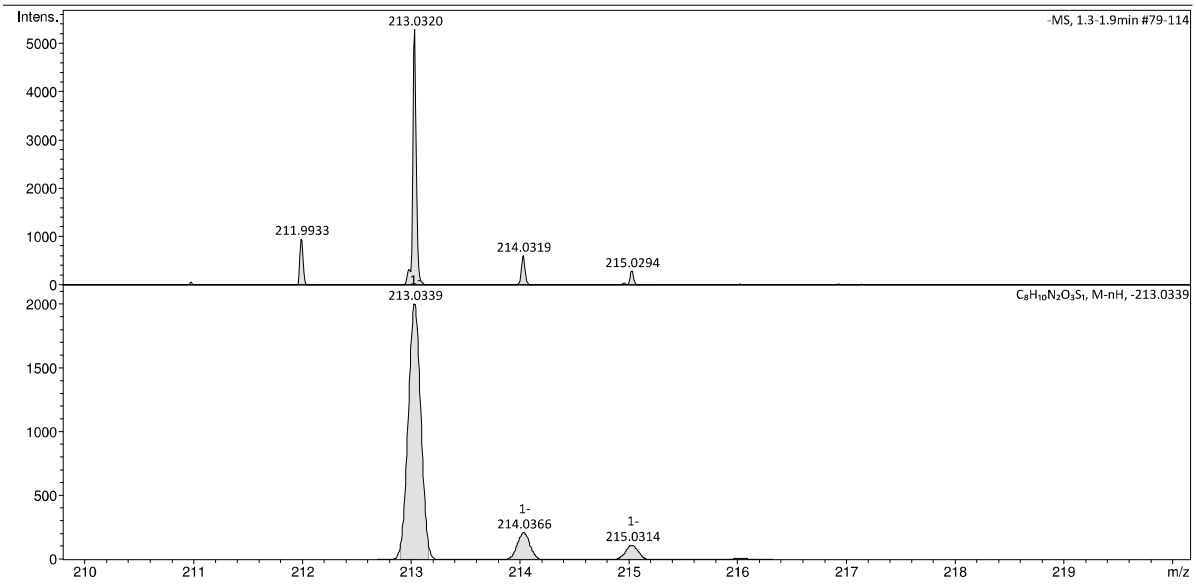
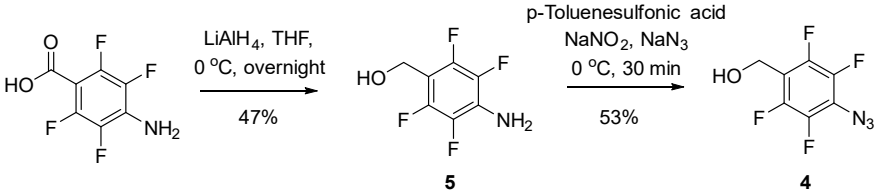


Fig. S6. HRMS spectrum of **2**, isolated from an irradiated mixture of **1** (60 Gy) by prep-HPLC. (ESI) for $C_8H_{10}N_2O_3S$ $[M-H]^-$: *calcd.*: 213.0339; found: 213.0320.

Synthesis of compound 4



Compound **5** and **4** was synthesised following reported procedure.[3]

Compound **5** was synthesised giving a brown solid in a 55% yield (950 mg).

Compound **5**, ^{1}H NMR (500 MHz, d-$CDCl_3$) δ (ppm) = 4.75 (d, J = 6.4, 2H), 4.05 (s, 2H), 1.81 (t, J = 6.3 Hz, 1H); ^{13}C NMR (125 MHz, d-$CDCl_3$) δ (ppm) = 144.34 (m, J_{C-F}), 136.05 (m, J_{C-F}), 126.43 (m, J_{C-F}), 105.73, 60.41; HRMS (ESI) for $C_7H_5F_4NO$ $[M+H]^+$: *calcd.*: 194.0203; found: 194.0234.

Compound **4** was synthesised giving a white solid in a 92% yield (99 mg).

Compound **4**, ^{1}H NMR (600 MHz, d-$CDCl_3$) δ (ppm) = 4.82 (d, J = 6.6 Hz, 2H), 1.92 (t, J = 6.6 Hz, 1H); ^{13}C NMR (150 MHz, d-$CDCl_3$) δ 144.43 (m, J_{C-F}), 140.72 (m, J_{C-F}), 120.02 (m, J_{C-F}), 114.07, 52.69.

Data in agreement with the literature.[3]

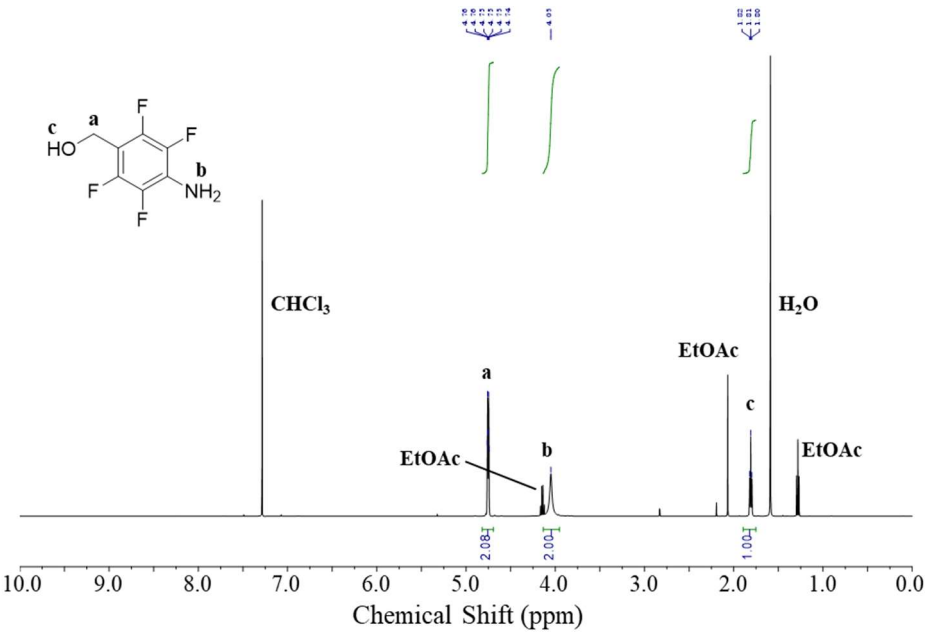


Fig. S7. ^{1}H NMR spectrum of model compound **5** in $CDCl_3$.

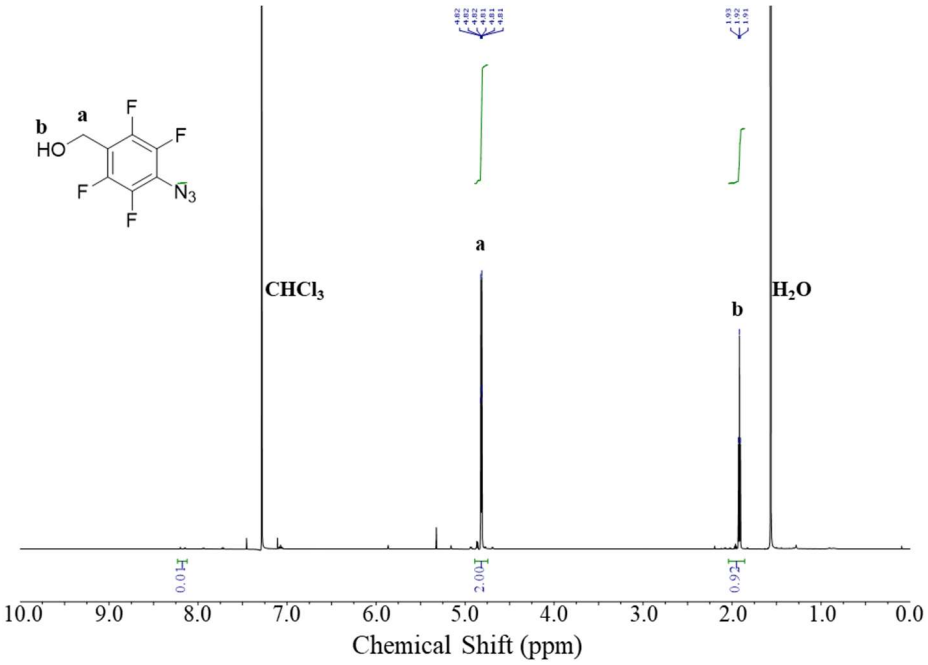
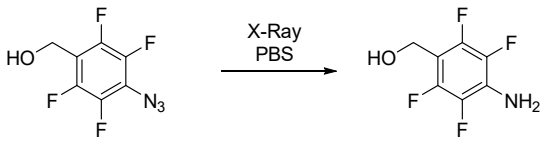


Fig. S8. ^{1}H NMR spectrum of model compound **4** in $CDCl_3$.

Reaction of model compound 4 under X-ray irradiation



4 **5**

A stock solution of model compound **4** (100 mM in DMSO) was diluted in PBS (20 mL) to give a final concentration of 10 μM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation. The isolated product was identified by ^{1}HNMR and HRMS.

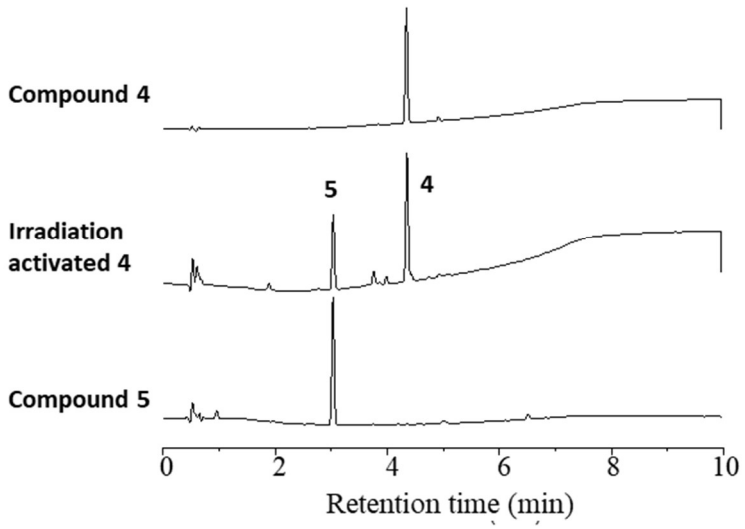


Fig. S9. HPLC traces of model compound **4**, irradiation activated **4** and compound **5** (synthesised as described above).

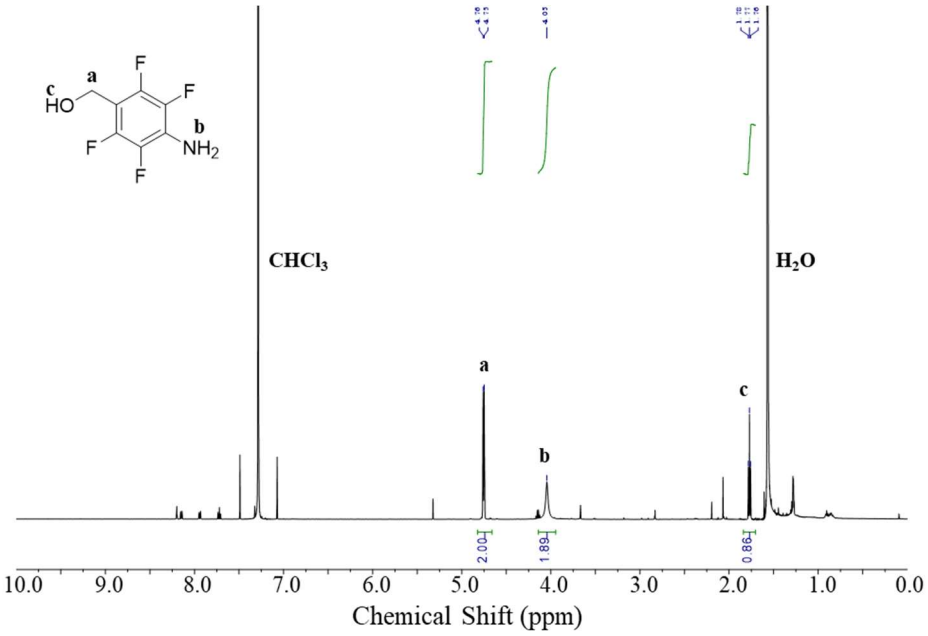


Fig. S10. ^{1}H NMR spectrum of **5** (in $CDCl_3$) isolated from irradiated **4** (60 Gy) by prep-HPLC.

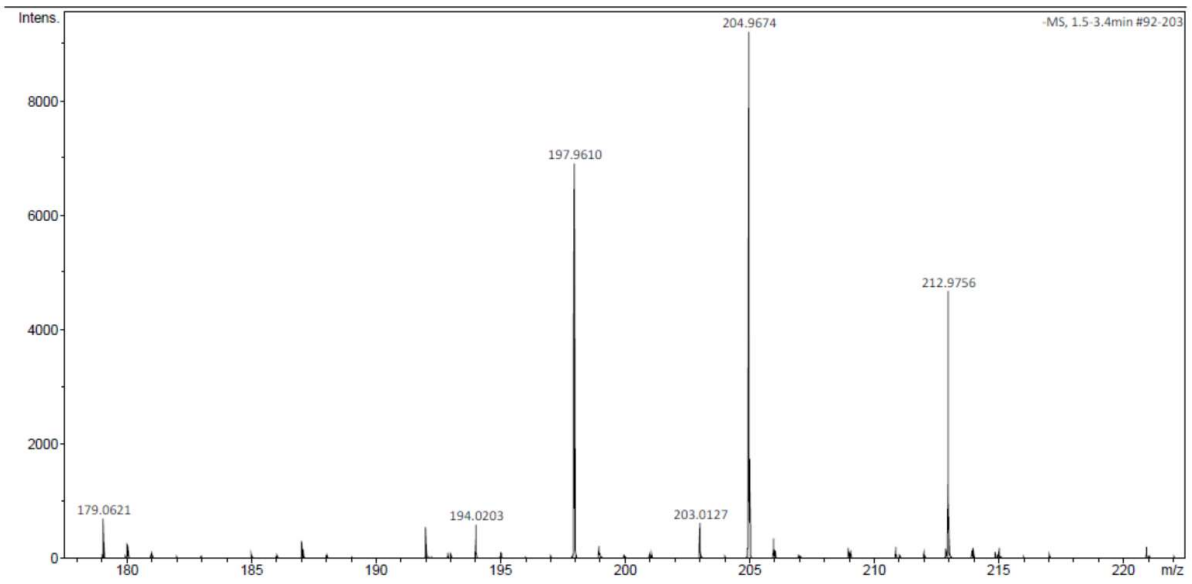
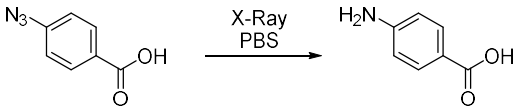


Fig. S11. HRMS analysis of **5**, isolated from irradiated **4** (60 Gy) by prep-HPLC. (ESI) for $C_7H_5F_4NO$ $[M-H]^-$: *calcd.*: 194.0236; found: 194.0203.

Model compound 6



6 **7**

A stock solution of model compound **6** (100 mM in DMSO) was diluted in PBS (20 mL) to give a final concentration of 10 μM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation. The product was identified by HPLC (identical to compound **7**, CAS 150-13-0).

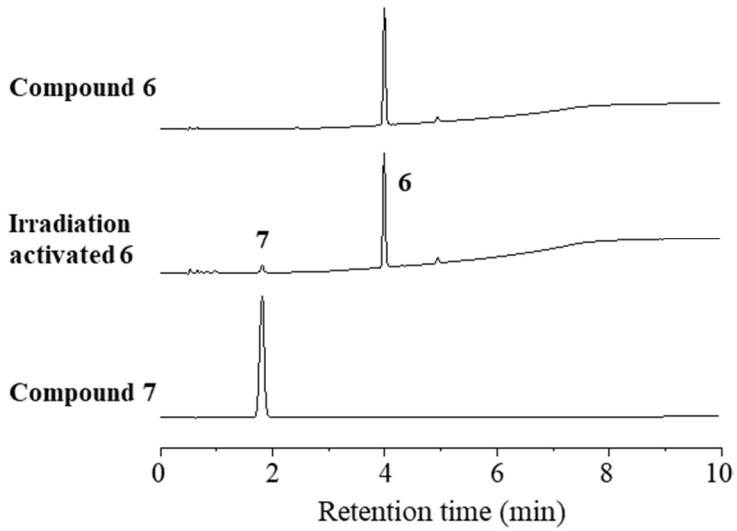


Fig. S12. HPLC traces of model compound **6**, irradiation activated **6** and compound **7** (obtained from Sigma-Aldrich).

Cell culture

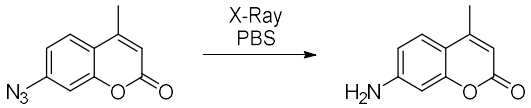
Hela cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), *L*-glutamine (4 mM) and antibiotics (penicillin and streptomycin, 100 units/mL). Cell culture was performed in a 5 % CO_2 atmosphere at 37 °C in a SteriCult 200 (Hucoa-Erloss) incubator. The cells were passaged using trypsin/EDTA (0.25 % trypsin, 1 mM EDTA) every 2 days.

HUVECs were maintained in 25 mm^3 tissue culture flasks (Corning) in F-12K medium, supplemented with 10 % (v/v) FBS, heparin (100 μg/mL, Sigma) and endothelial cell growth supplement (BD Biosciences) in a cell incubator (37 °C and 5 % CO_2). The cells were passaged using trypsin/EDTA every 5 days.

Evaluation of compound cytotoxicity

Compound cytotoxicities were evaluated using an MTT assay. Briefly, Hela and HEVEC cells were seeded in 96-well plates (1×10^4 cells/well) and incubated overnight. The cells were treated with the desired compound at different concentrations in DMEM for 24 h. The media was removed and the cells were washed with PBS (× 3) and incubated with 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/mL) for 4 h at 37 °C. 100 µL of MTT solubilisation solution (10% Triton-X 100 in 0.1 N HCI in isopropanol) was added to each well and the plate was shaken horizontally for 60 min to dissolve the formazan crystals. The absorbances at 570 nm were measured on a multimode plate reader and cell viability was calculated compared to untreated cells.

Reaction of coumarin azide 8 under X-ray irradiation



8 **9**

A stock solution of coumarin azide **8** (1 M in DMSO) was diluted in PBS (20 mL) to a final concentration of 100 µM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation. The fluorescence intensity of the reaction mixtures was analysed. The product **9** was isolated by prep-HPLC and characterised by ^{1}HNMR and HRMS.

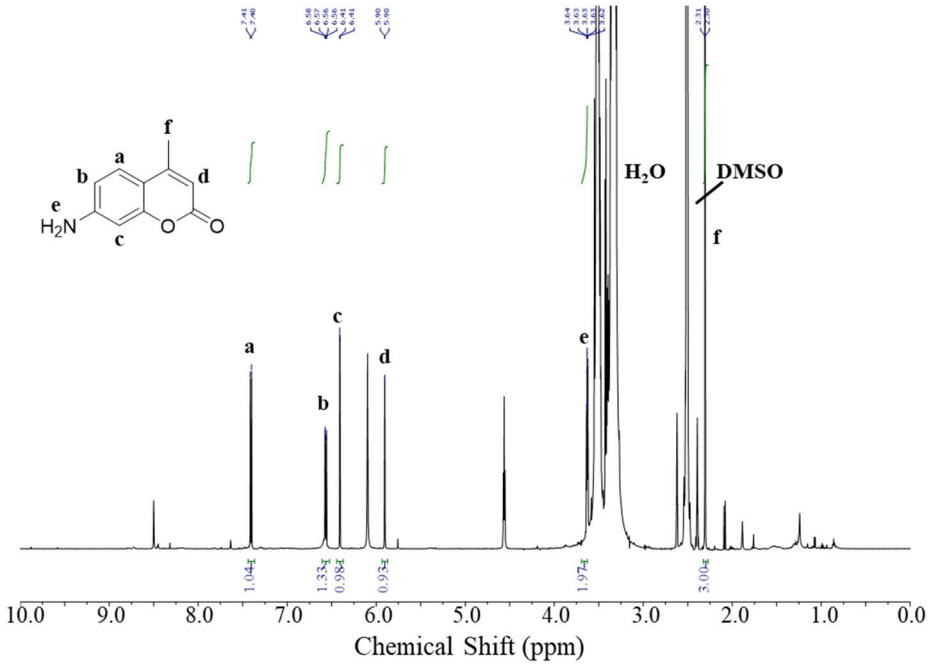


Fig. S13. ^{1}H NMR spectrum of **9** (in d_6-DMSO) purified from irradiated **8** (60 Gy) by prep-HPLC.

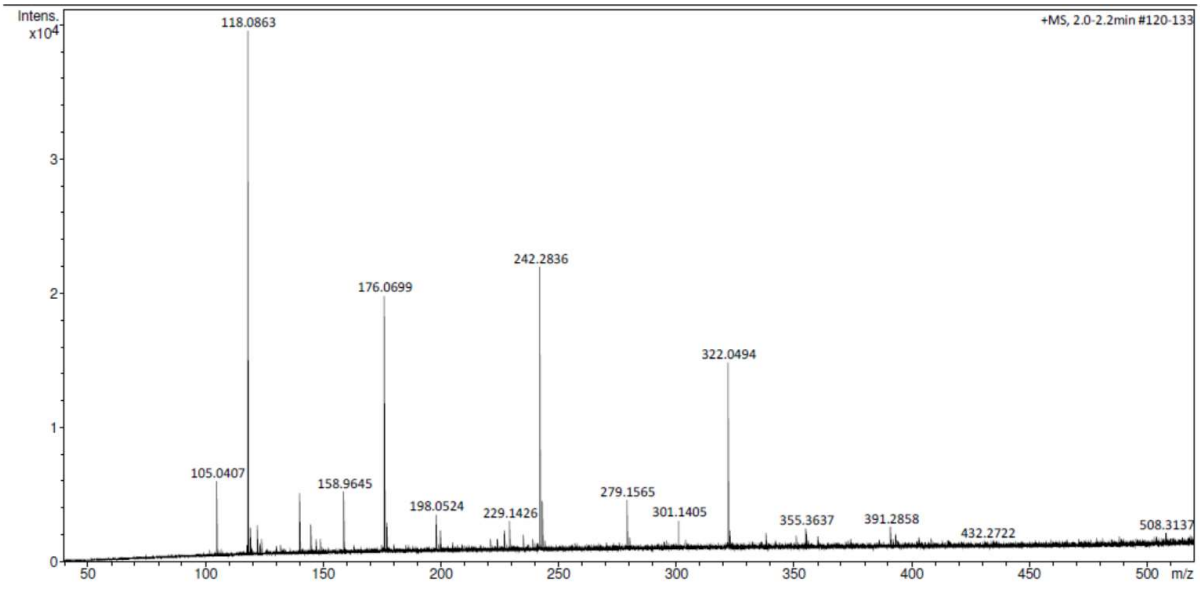


Fig. S14. HRMS spectrum of **9**, isolated from irradiated **8** (60 Gy) by prep-HPLC. (ESI) for $C_{10}H_9NO_2$ $[M+H]^+$: *calcd.*: 176.0706; found: 176.0699.

Quantification of the reaction of coumarin azide 8 under X-ray irradiation

The coumarin azide PBS solutions (100 μM) were irradiated with X-rays with a dose of 0 – 60 Gy. The fluorescence spectra were recorded using a FluoroMax-3 Jobin Yvon Div fluorimeter using a quartz cuvette (λ_{ex} = 345 nm). An aliquot of each solution was analysed by HPLC and the conversion of the azide was quantified by integration of the HPLC peaks (using a calibration line generated using solutions of known concentrations).

Reaction of coumarin azide 8 in live cells under X-ray irradiation

Hela cells were seeded in 24-well plates at a density of 5 × 10^4 cells per well and incubated overnight. The cells were then treated with coumarin azide **8** (100 μM) 1 h prior to irradiation (0, 6, 36 and 60 Gy) and analysed by flow cytometry using a DAPI filter ($\lambda_{ex/em}$ = 360/450 nm) and confocal microscopy.

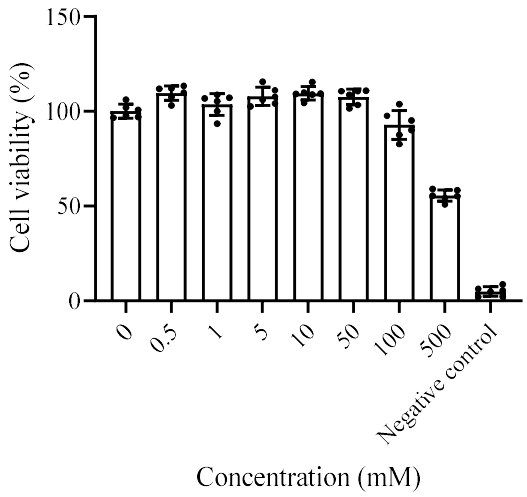


Fig. S15. Hela cell viability against coumarin azide **8** (MTT assay, error bars represent standard deviation, n = 6). As a negative control, the cells were treated with 50% DMSO in DMEM. The data are presented as mean ± SD (n = 3). The experiments were repeated, independently, 3 times with similar results observed.

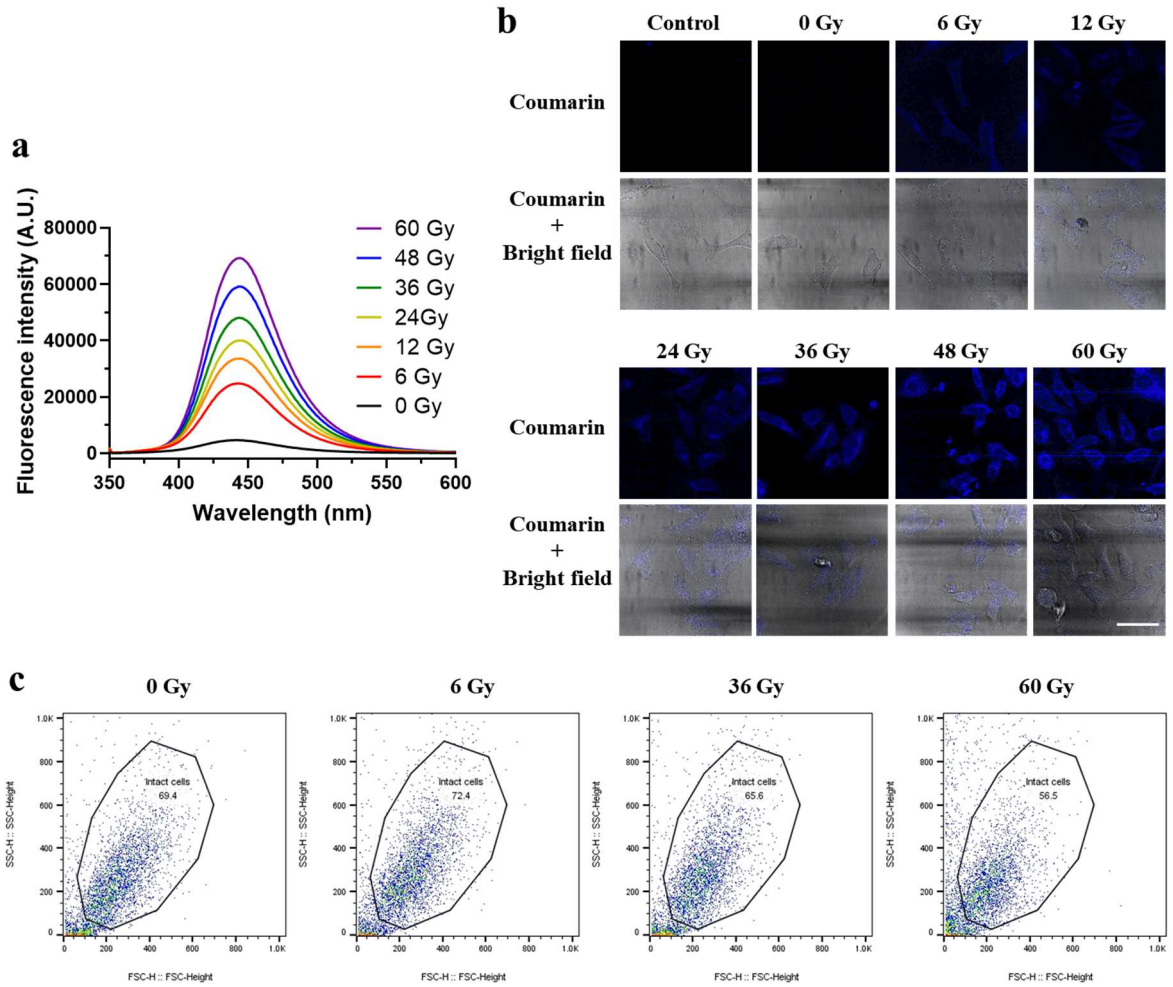
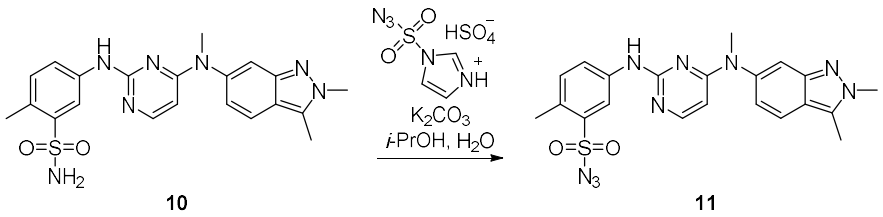


Fig. S16. (**a**) Fluorescence spectrum of **8** (100 μM in PBS, pH = 7.4, $\lambda_{ex/em}$ = 345/443) with irradiation with 0 Gy to 60 Gy with the fluorescence intensity increasing with irradiation dose. (**b**) Confocal fluorescence microscopy images of HeLa cells, with and without irradiation, showing decaged fluorescent 7-amino-4-methylcoumarin **9** (blue, $\lambda_{ex/em}$ = 360/450 nm) in cells (untreated Hela cells were used as control). Scale bar = 50 μm. (**c**) Graphical account of the gating strategy for the flow cytometry analysis of cells incubated with **8** (100 μM). Forward versus side scatter profiles were used to gate intact cellular materials. Flow cytometry analyses are based on a population of 10,000 cells. The experiments were repeated, independently, 4 times with similar results observed.

Synthesis of prodrug 7



10 11

Pazopanib **10** (218 mg, 0.5 mmol) and K_2CO_3 (276 mg, 2 mmol) were dissolved in 6 mL of a 1:1 mixture H_2O/*i*-PrOH to which was then added imidazole-1-sulfonylazide hydrochloride (157 mg, 0.75 mmol). After being stirred for 18 h at ambient temperature, the reaction mixture was diluted with saturated $NaHCO_3$ (30 mL) and extracted with EtOAc (2 × 60 mL). The combined organic phases were washed twice with brine and dried over Na_2SO_4 and the volatiles evaporated *in vacuo*. The residue was purified by silica gel chromatography (5% MeOH in DCM) to afford the prodrug **11** as a white solid (151 mg, 65%): ^{1}H NMR (500 MHz, d_6-DMSO) δ (ppm) = 8.87 (d, *J* = 2.4 Hz 1H), 7.88-7.85 (m, 2H), 7.77 (d, *J* = 8.8 Hz, 1H), 7.46 (s, 1H), 7.39 (d, *J* = 8.4 Hz, 1H), 6.90 (m, 1H), 5.78 (d, *J* = 2.4 Hz 1H), 4.06 (s, 3H), 3.50 (s, 3H), 2.63 (s, 3H), 2.52 (s,3H); ^{13}C NMR (125 MHz, d_6-DMSO) δ (ppm) = 162.40, 159.13, 155.76, 146.96, 141.74, 140.02, 135.66, 133.37, 132.21, 128.47, 124.36, 121.93, 119.55, 117.94, 114.05, 97.14, 48.59, 37.97, 37.38, 18.98, 9.41.; IR (solid) cm^{-1}: 2934, 2848, 2123, 1734, 1614, 1573,, 1517,1423, 1402, 1361, 1234, 1163; HRMS (ESI) for $C_{21}H_{21}N_9O_2S$ $[M+H]^+$: *calcd*.: 464.1612; found: 464.1604.

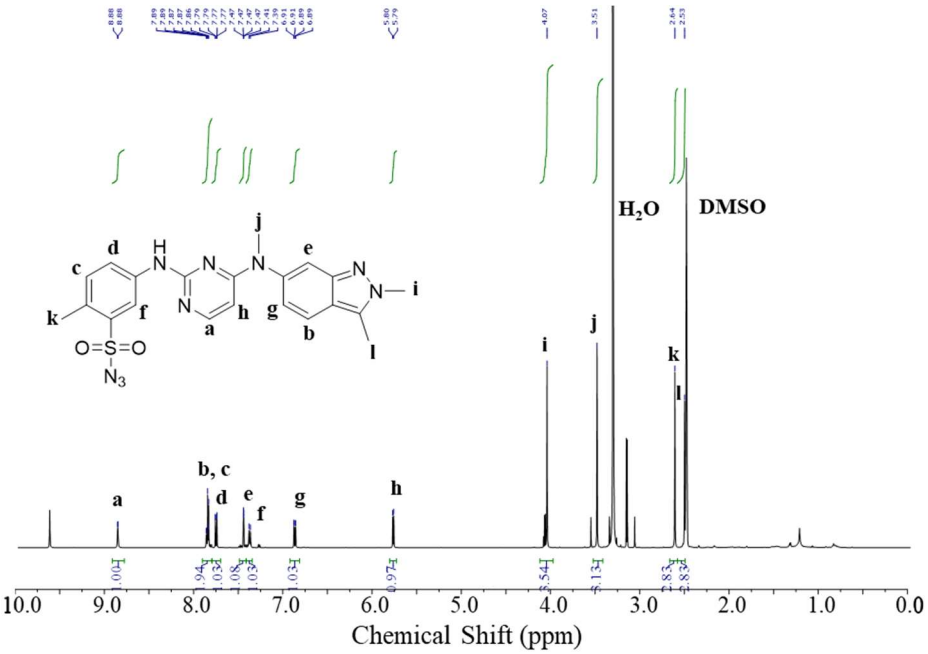


Fig. S17. ^{1}H NMR spectrum of prodrug **11** in d_6-DMSO.

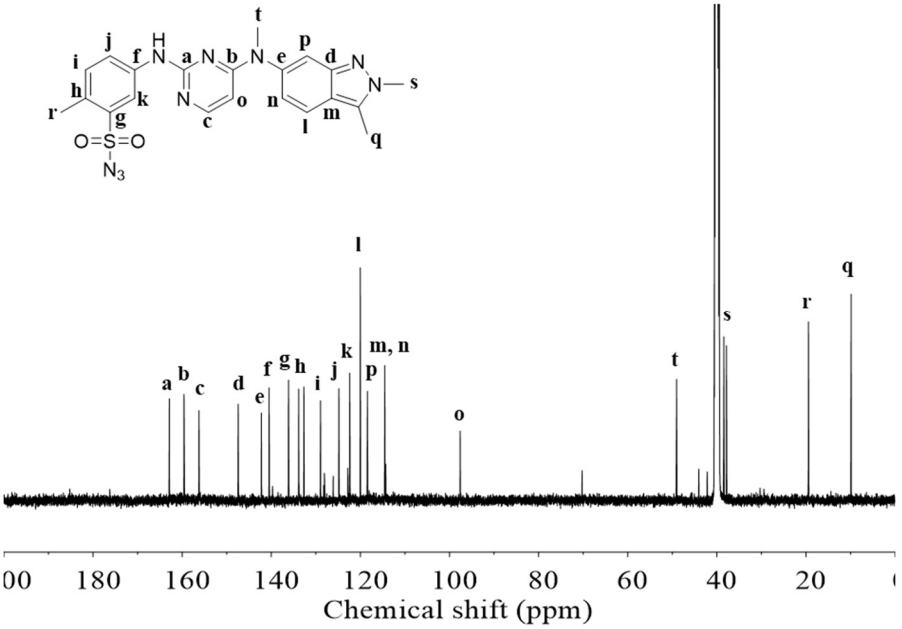
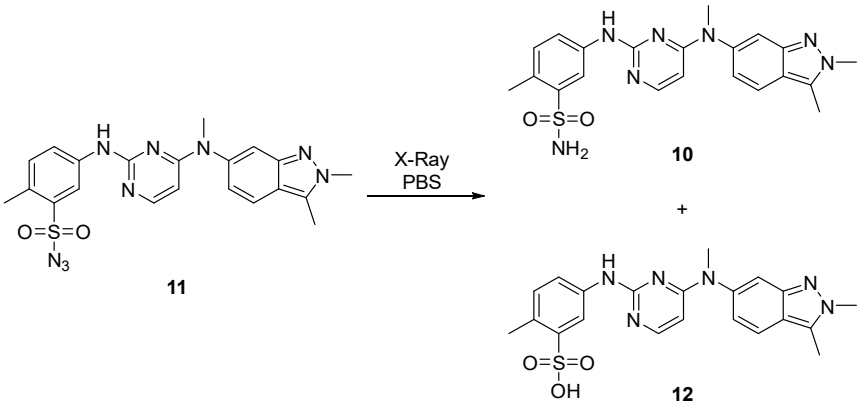


Fig. S18. ^{13}C NMR spectrum of prodrug **11** in d_6-DMSO.

Reaction of prodrug 11 under X-ray irradiation



A stock solution of prodrug **11** (100 mM in DMSO) was diluted in PBS (20 mL) to a final concentration of 20 µM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation. The reaction mixture was analysed by FTIR and the products **10** and **12** were isolated by prep-HPLC and characterised by ^{1}HNMR and HRMS.

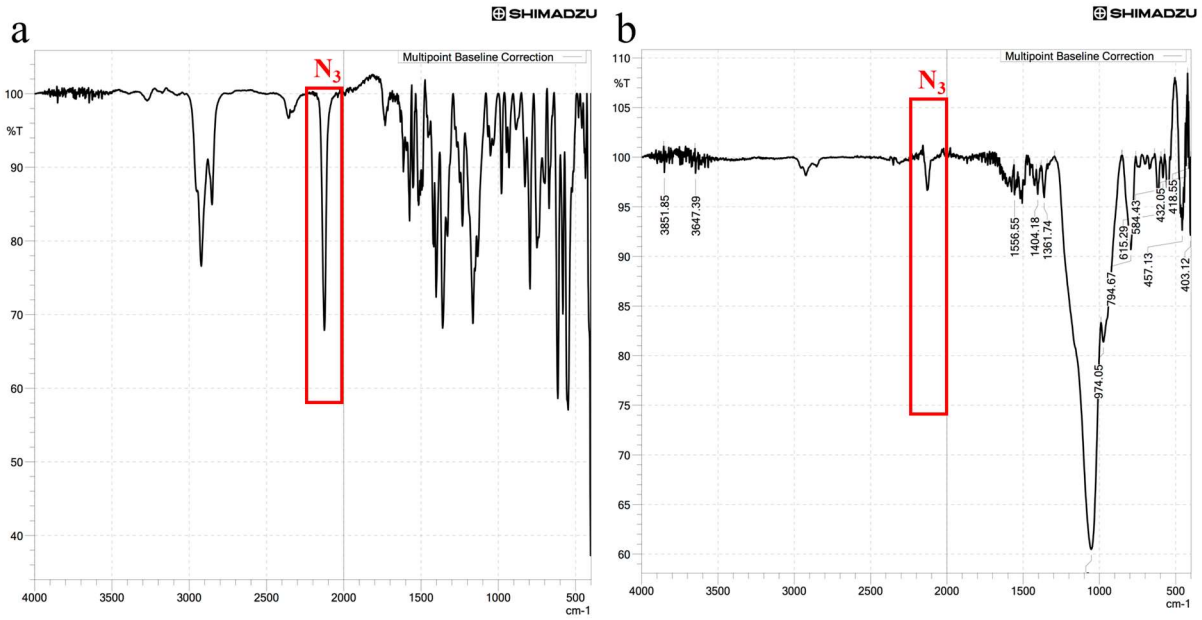


Fig. S19. FTIR spectrum of compound (a) prodrug **11** and (b) irradiated prodrug **11** (60 Gy). The decreased intensity of the azide signal (2134.1 cm^{-1}) indicated the consumption of prodrug **11**.

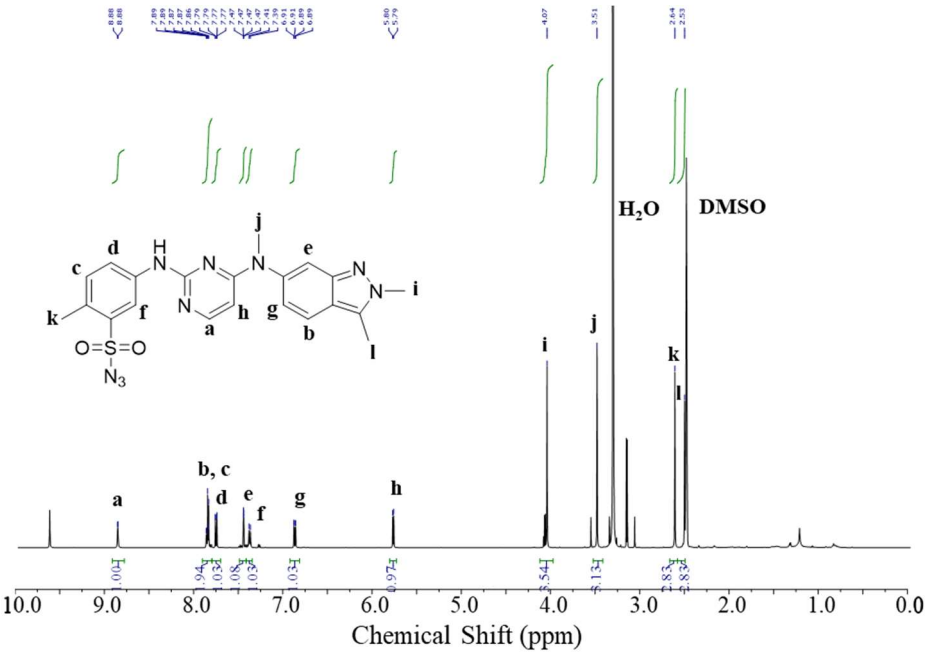


Fig. S20. ^{1}H NMR spectrum of **10** (in d_4-MeOD) f purified from irradiated prodrug **11** (60 Gy) by prep-HPLC.

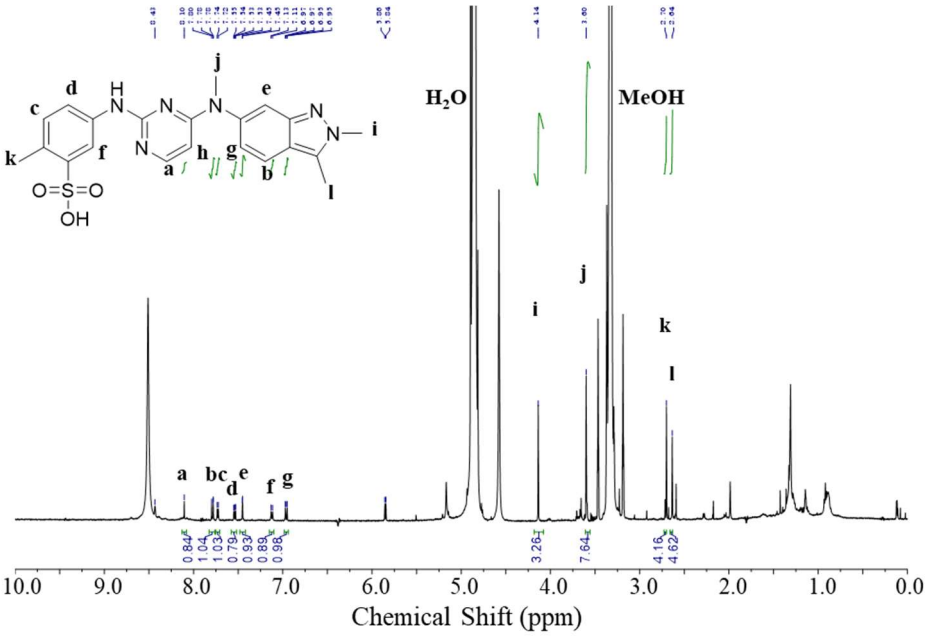


Fig. S21. ^{1}H NMR spectrum of **12** (in d_4-MeOD) isolated from irradiated prodrug **11** (60 Gy) by prep-HPLC.

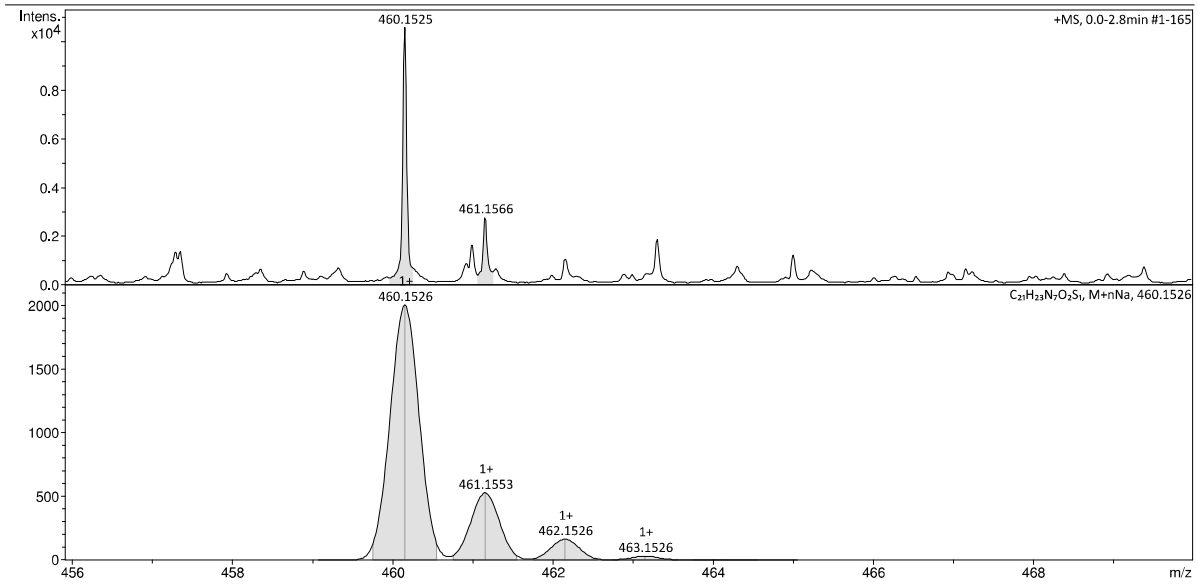


Fig. S22. HRMS spectrum of **10**, isolated from an irradiated prodrug **11** (60 Gy) by prep-HPLC. (ESI) for $C_{21}H_{23}N_7O_2S$ $[M+Na]^+$: *calcd.*: 460.1526; found: 460.1525.

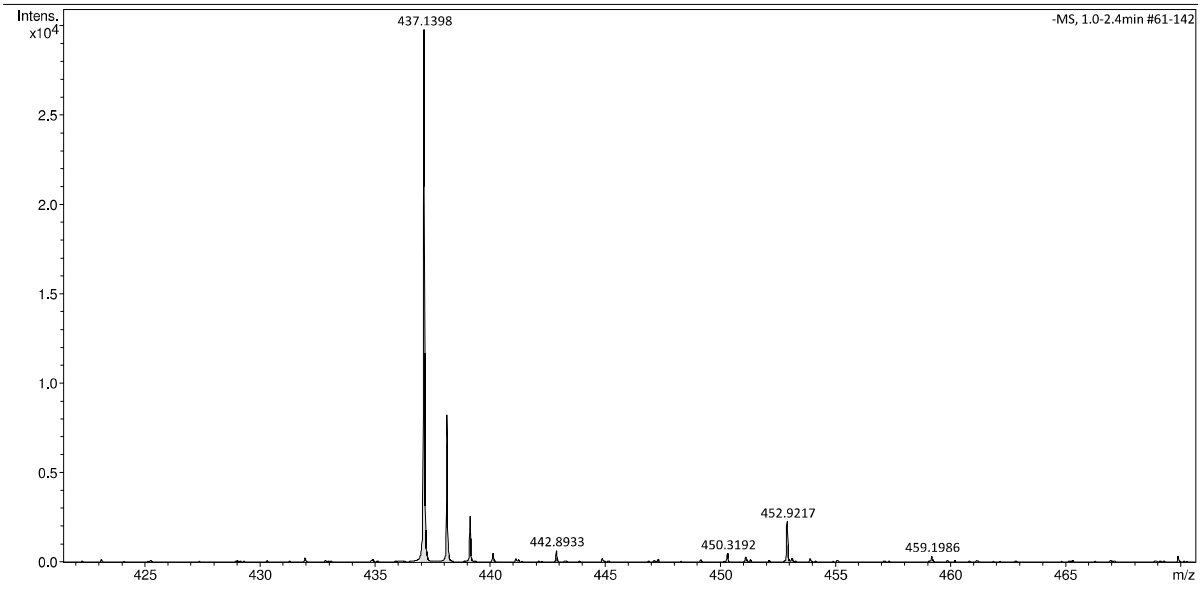


Fig. S23. HRMS of **12**, isolated from irradiated prodrug **11** (60 Gy) by prep-HPLC. (ESI) for $C_{21}H_{22}N_6O_3S$ $[M-H]^-$: *calcd.:* 437.1401; found: 437.1398.

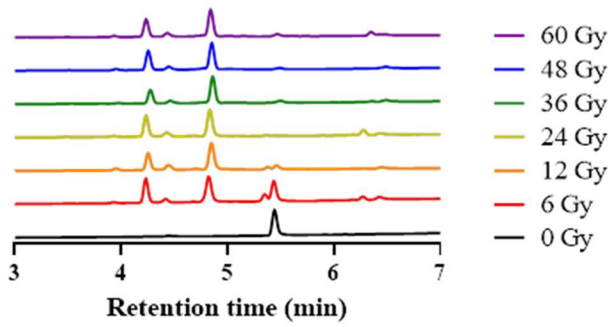


Fig. S24. HPLC traces of the reaction mixture of prodrug **11** (R_T = 5.44 min) after irradiation with 6 Gy to 60 Gy (without degassing). The reaction afforded product **10** (R_T = 4.23 min) and **12** (R_T = 4.83 min) with some remaining unreacted prodrug **11**.

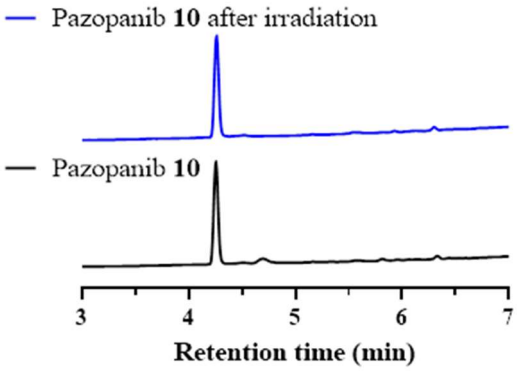


Fig. S25. HPLC traces (254 nm) of pazopanib **10** before and after X-ray irradiation (60 Gy).

Cell viability upon treatment of the pazopanib 10 and prodrug 11 before and after X-ray irradiation

Viability of HUVEC were evaluated by a Celltiter Glo® assay. HUVECs were seeded in an opaque-walled 96-well plate at a density of 1×10^4 cells per well and incubated overnight. The cells were then treated with pazopanib **10**, prodrug **11** and irradiated prodrug **11** (60 Gy) at concentrations of 5, 10 and 20 µM for 24, 48 h. The medium was replaced with fresh medium and 100µl of CellTiter-Glo® 2.0 reagent was added directly to the culture media and the content was mixed for 2 minutes on an orbital shaker followed by 10 min incubation at RT. The

luminescence was measured by a plate reader. Cell viability was calculated compared to untreated cells. All of the experiments were repeated three times.

Cell proliferation assay of pazopanib 10 and prodrug 11 before and after X-ray irradiation

Proliferation of HUVEC were evaluated by a Click iT® EdU assay. Briefly, HUVECs were seeded in a 24-well plate at a density of 5×10^4 cells per well and incubated overnight. The cells were then treated with pazopanib **10**, prodrug **11** and irradiated prodrug **11** at concentrations of 10 and 20 μM for 24 h and washed gently with PBS before fresh media were added. A solution of EdU was added (10 μM) to each well and incubated for 4 h. The cells were harvested and washed with PBS (containing 1% BSA) before permeabilisation with saponin (0.1% w/v) and fixation with 4% PFA. The fixed cells were treated with Click-iT® reaction cocktail (1 ×), incubated for 30 min, washed with PBS (containing 1% BSA) and analysed by flow cytometry using a FITC filter ($\lambda_{ex/em}$ = 488/525 nm).

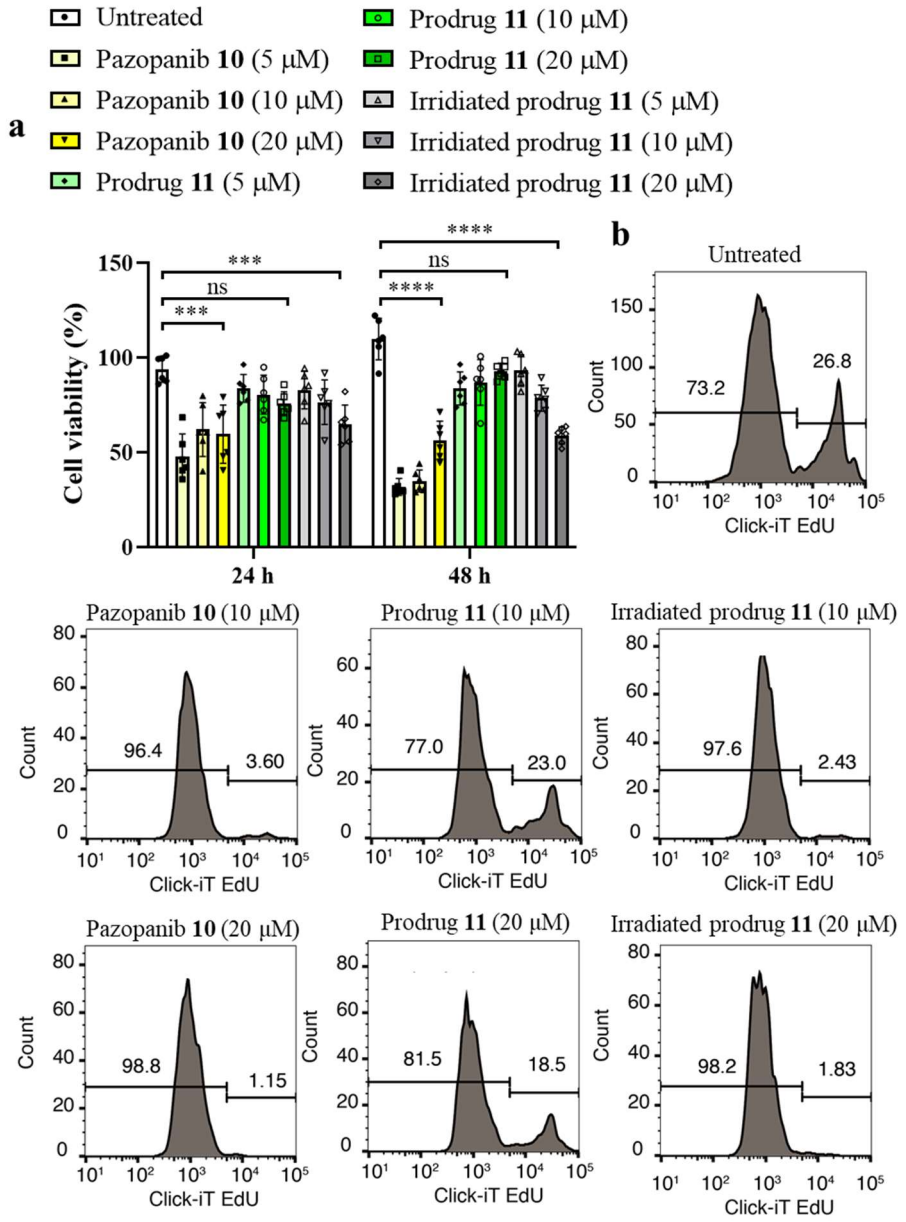


Fig. S26. HUVECs viability and proliferation as affected by pazopanib **10** and irradiation activated prodrug **11**. (**a**) Hela cell viabilities were analysed using a Celltiter Glo® assay. The cells (1×10^4 per well) were treated with pazopanib **10** prodrug **11** and irradiation activated prodrug at the concentrations of 5 μM, 10 μM and 20 μM for 24 hours and 48 hours at 37 ºC. Data represent the mean ± SD, n = 6 independent experiments. Significant differences were analysed using one-way analysis of variance followed by Sidak's multiple comparison test compared to an untreated control group (ns, not significant, *** $P = 0.0003$, **** $P < 0.0001$). (**b**) Proliferation of HUVEC were evaluated by Click-iT® EdU assay. The cells (5×10^4 per

well) were treated with pazopanib **10** prodrug **11** and irradiation activated prodrug at the concentrations of 10 μM and 20 μM for 24 hours and analysed by flow cytometry using a FITC filter ($\lambda_{ex/em}$ = 488/525 nm). Forward versus side scatter profiles were used to gate-in intact cellular materials (remove cell debris). Flow cytometry analyses are based on a population of 10,000 cells. The experiments were repeated, independently, 3 times with similar results observed.

Long term viability affected by pazopanib 10 and prodrug 11 before and after X-ray irradiation

Long term viability of HUVEC was evaluated by Celltiter Glo® assay. HUVECs were seeded in an opaque-walled 96-well plate at a density of 1×10^4 cells per well and incubated overnight. The cells were then treated with prodrug **11** at 0, 5 and 10 μM for 4 h and irradiated with x-ray for 0, 6, 12 and 24 Gy. The medium was replaced and cells were incubated in fresh media for 24, 48 and 72 h, and 100μl of CellTiter-Glo® 2.0 reagent was added directly to the culture media and the content was mixed for 2 minutes on an orbital shaker followed by 10 min incubation at RT. Luminescence was measured by a plate reader (n = 6). The experiments were repeated three times.

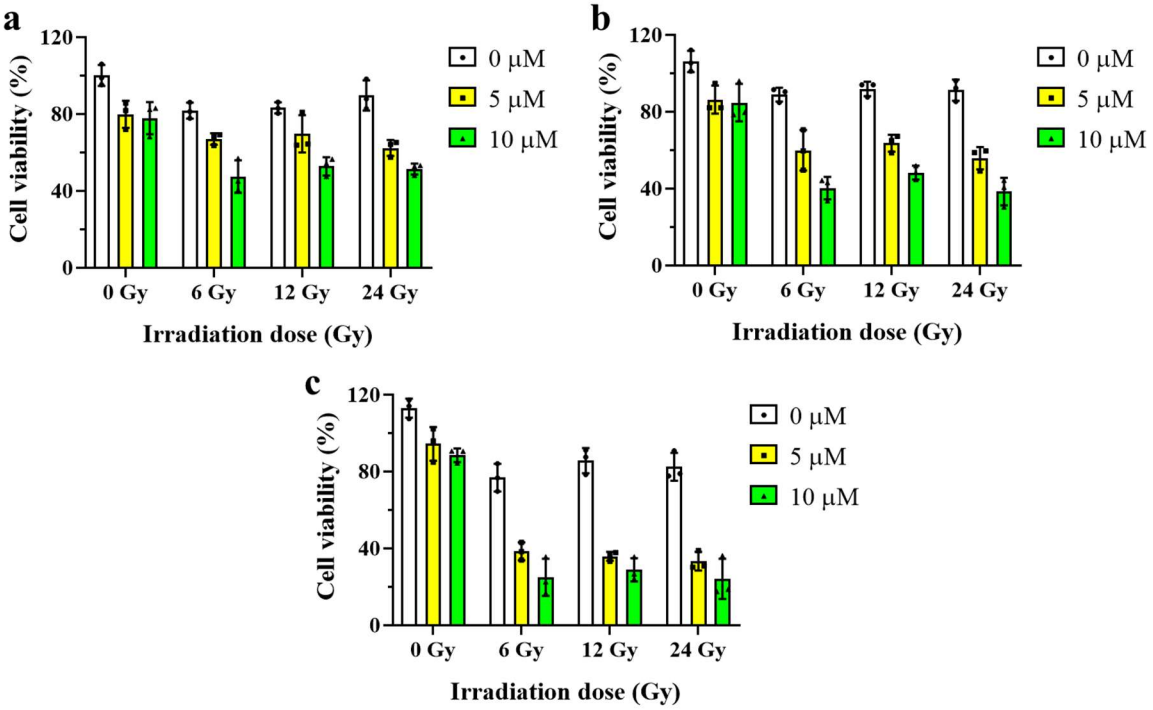


Fig. S27. (**a**) 24 h, (**b**) 48 h and (**c**) 72 h HUVECs viability (Celltiter Glo® assay) following exposure to prodrug **11** with different irradiation doses. Data represent the mean ± SD, n = 3 independent experiments. The experiments were repeated, independently, 5 times with similar results observed.

Transwell migration assay for pazopanib 10 and prodrug 11 before and after X-ray irradiation

HUVECs were starved by incubation in Endothelial Cell Growth Medium (serum free, Sigma) containing 0.1% fatty acid-free BSA for 4 h prior to the experiment. Serum free endothelial cell growth media (25 μL) with and without addition of VEGF (100 ng/mL) to the bottom well and the starved cells were harvested into the same medium and re-seeded in the top insert of a Corning® HTS Transwell® 96 well permeable supports at a density of 2×10^5 cells per well and treated with pazopanib **10**, prodrug **11** and irradiated prodrug **11** at concentrations of 5 μM, 10 μM and 20 μM for 24 h. The top insert was removed and the migrated cells in bottom wells were washed with PBS for 3 times and stained with Hoechst 33342 before fixation with 4%

PFA and imaged using the bright field channel and DAPI (λ_{ex} = 340-395 nm, λ_{em} = 430-505 nm) channel on a Zeiss AxioVert 200M fluorescent microscope. Cells were counted based on their nuclear staining and the cell morphology.

Wound healing assay for pazopanib 10 and prodrug 11 before and after X-ray irradiation

HUVECs were seeded in Ibidi® Culture-Insert 2 Well μ-Dish at a density of 2×10^4 cell per well (in 70 μL media) and incubated at 37 °C overnight. To each well, pazopanib **10**, prodrug **11** and irradiated prodrug **11** at concentrations of 10 and 20 μM were added and incubated at 37 ˚C for 24 h, followed by gentle washing with PBS (3 × 70 μL). Fresh media (70 μL) was added and the insert modes were removed, followed by addition of fresh media (2 mL) to the dish. Bright field microscopy images were taken at t = 0, 2h, 4h and 6h, and the width of the void between cells measured using ImageJ with the "Wound Healing Tool" plugin, using the variance method.

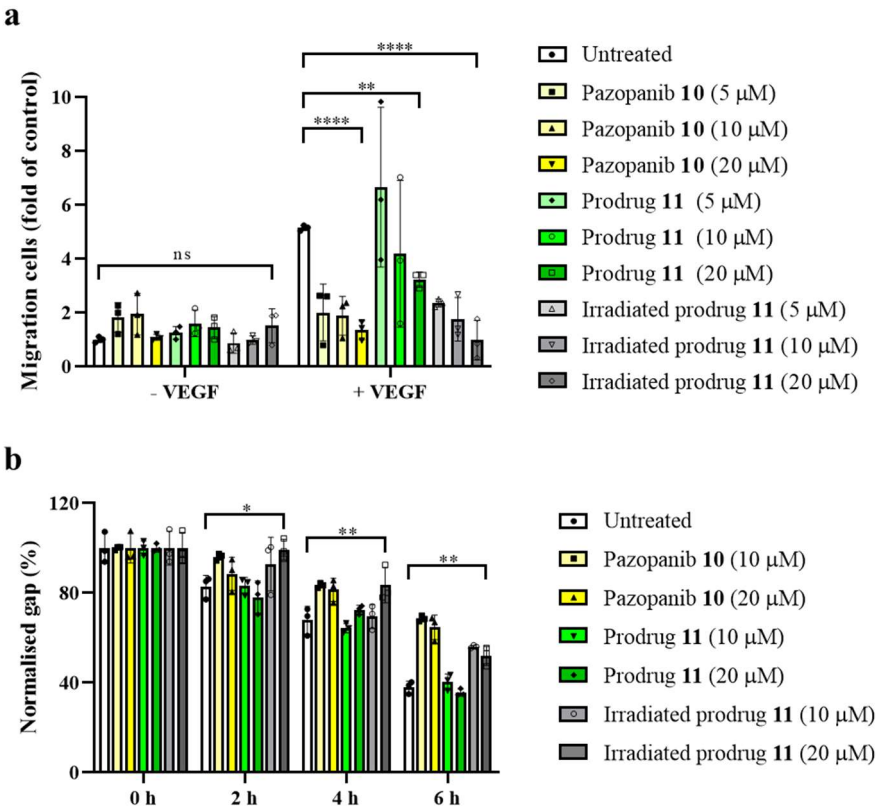


Fig. S28. (**a**) Transwell migration assay of HUVEC cells treated with pazopanib **10**, prodrug **11** and irradiated prodrug. The data are presented as mean ± SD (n = 3 independent samples for each group). Significant differences were analysed using one-way analysis of variance followed by Sidak's multiple comparison test compared to an untreated control group (ns, not significant, ** $P = 0.0075$, **** $P < 0.0001$). (**b**) Wound healing experiments of HUVEC cells. The normalised gaps *vs* time (calculated as the ratio of the remaining gap area at the given time point and at t = 0 h). Data represent the mean ± SD, n = 3 independent experiments. Significant differences were analysed using one-way analysis of variance followed by Sidak's multiple comparison test compared to an untreated control group (* $P = 0.0135$, ** $P = 0.0021$). The experiments were repeated, independently, 3 times with similar results observed.

Endothelial tubule formation assay for pazopanib 10 and prodrug 11 before and after X-ray irradiation

HUVECs were starved by incubation in Endothelial Cell Growth Medium (serum free, Sigma) containing 0.1% fatty acid-free BSA for 4 h prior to the experiment. The starved cells were harvested into the same medium and re-seeded in a 96-well plate coated with Matrigel Matrix(Corning) at a density of 5×10^3 cells per well, treated with prodrug **11** at concentrations of 0, 5 and 10 μM and irradiated with X-ray at 0, 6, 12 and 24 Gy. Tube formation was assessed by photography using the bright field channel 24 h after plating.

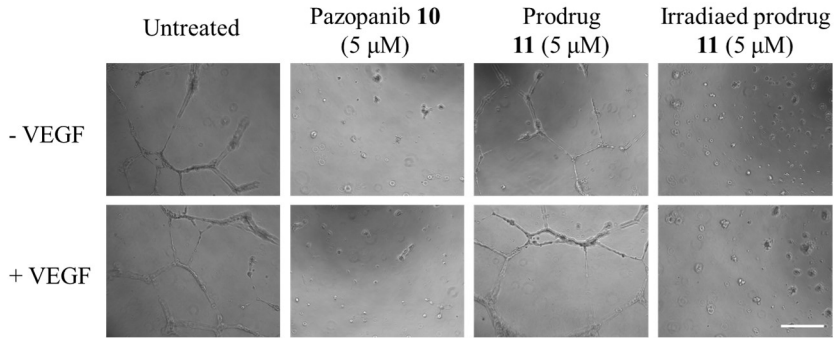


Fig. S29. HUVEC tubule formation affected by pazopanib **10**, prodrug **11**, and irradiated prodrug **11**, with and without supplement of VEGF. Scale bar = 200 μm. The experiments were repeated, independently, 3 times with similar results observed.

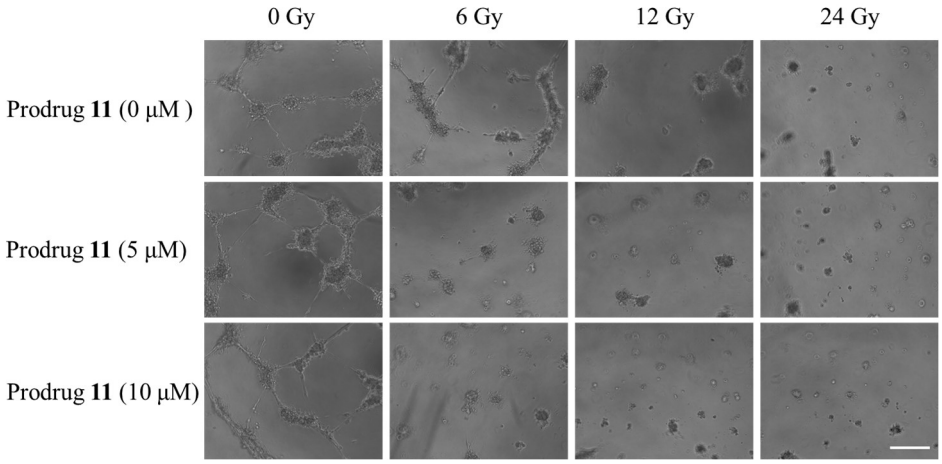
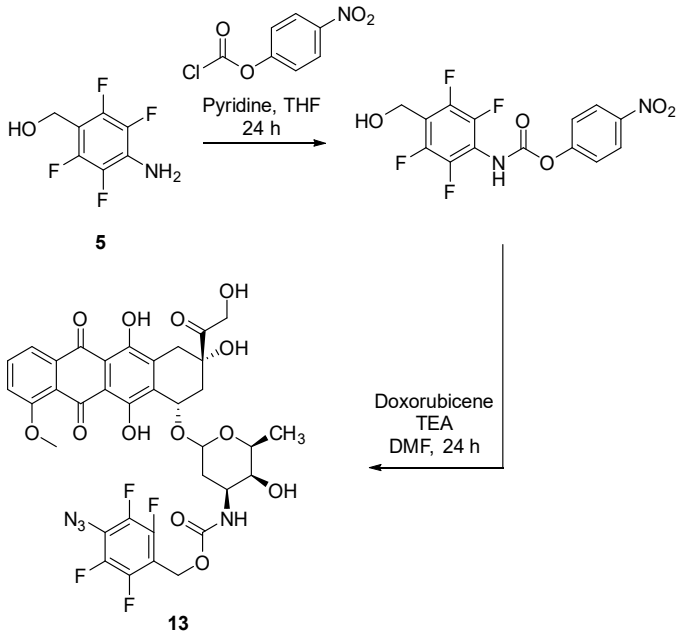


Fig. S30. HUVEC tubule formation affected by pazopanib **10**, prodrug **11**, and irradiated prodrug **11** with different irradiation dose. Scale bar = 200 μm. The experiments were repeated, independently, 3 times with similar results observed.

Synthesis of doxorubicin prodrug 13



Compound **13** was synthesised following a reported procedure giving a dark red solid in an 81% yield (39 mg).[3]

^{1}H NMR (500 MHz, d-$CDCl_3$) δ (ppm) = 14.02 (s, 1H), 13.30 (s, 1H), 8.08 (d, *J* = 7.7 Hz, 1H), 7.82 (dd, *J* = 8.5, 7.7 Hz, 1H), 7.43 (dd, *J* = 8.5, 1.1 Hz, 1H), 5.52 (d, *J* = 4.0 Hz, 1H), 5.32 (s,

1H), 5.15 (s, 2H), 5.11 (m, 1H), 4.78 (m, 2H), 4.51 (s, 1H), 4.17 (m, 1H), 4.12 (s, 3H), 3.88 (s, 1H), 3.68 (s, 1H), 3.36 – 3.29 (m, 1H), 3.00 (m, 1H), 2.36 (d, J = 14.8 Hz, 1H), 2.24 – 2.16 (m, 1H), 1.91 (m, 2H), 1.79 (m, 1H), 1.31 (d, J = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, d-$CDCl_3$) δ (ppm) = 213.3, 186.2, 186.1, 169.6, 156.2, 155.8, 154.7, 145.4, 140.2, 135.3, 135.2, 120.5, 119.7, 118.7, 110.1, 100.8, 77.3, 76.0, 69.9, 69.8, 69.4, 67.2, 65.2, 56.7, 56.6, 53.9, 46.8, 35.6, 33.9, 29.9, 16.9, 16.8; HRMS (ESI) for $C_{35}H_{30}F_4N_4O_{13}$ $[M+Na]^+$: *calcd.*: 813.1638; found: 813.1632. Data were in agreement with the literature.[3]

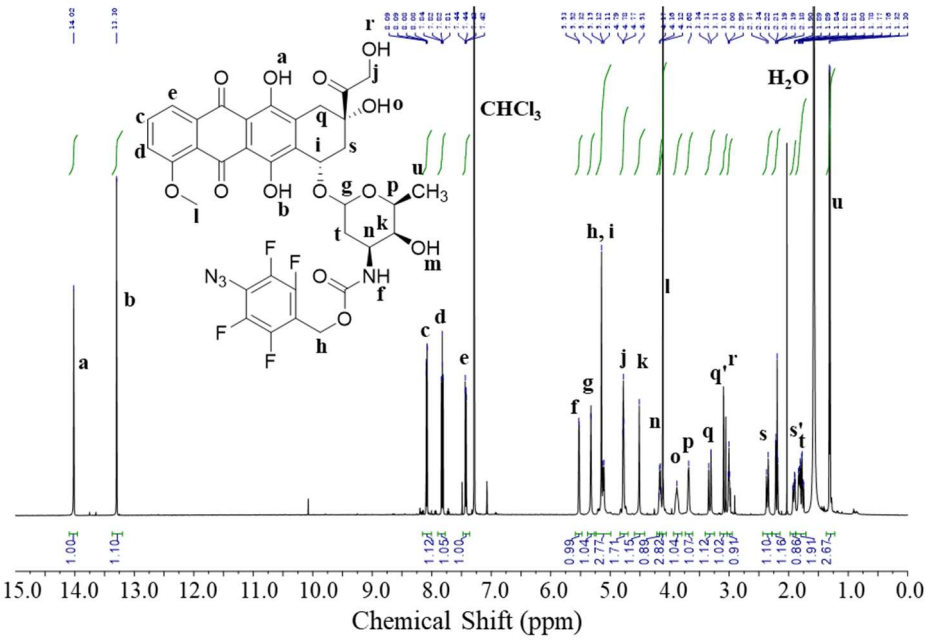
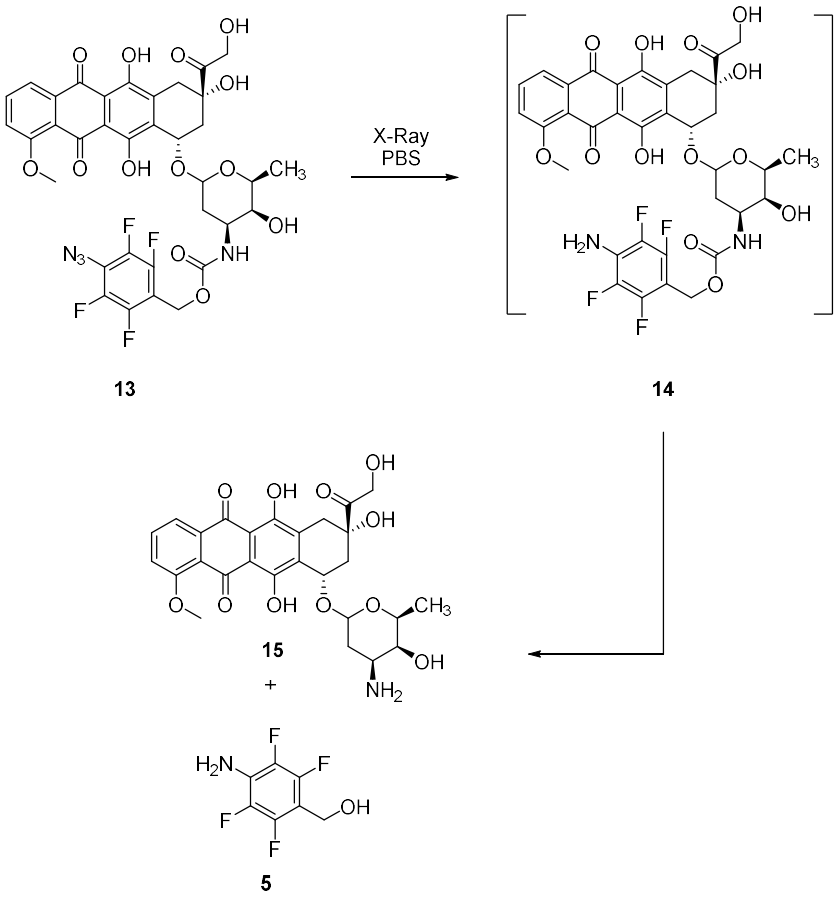


Fig. S31. ^{1}H NMR spectrum of prodrug **13** in $CDCl_3$.

Reaction of prodrug 13 under X-ray irriadation



A stock solution of doxorubicin prodrug **13** (100 mM in DMSO) was diluted in PBS (20 mL, with 0.1%, *v*/*v* Triton X100) to give a final concentration of 20 μM. The solution was degassed by bubbling Ar for 30 min before X-Ray irradiation. The reaction mixture was analysed by FTIR and the products doxorubicin **15** and compound **5** were isolated by prep-HPLC and characterised ^{1}HNMR and HRMS. (Note compound **15** and **5** co-eluted on the HPLC).

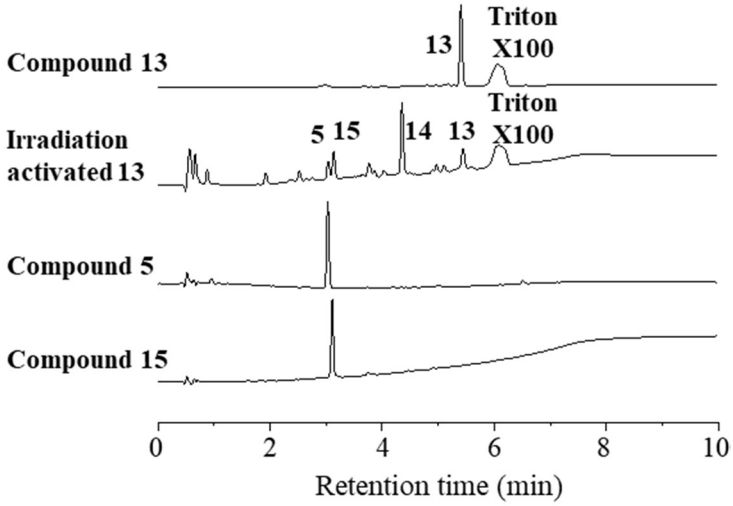


Fig. S32. HPLC traces of doxorubicin prodrug **13**, irradiation activated prodrug **13**, compound **5** and doxorubcin **15** (obtained from Sigma-Aldrich).

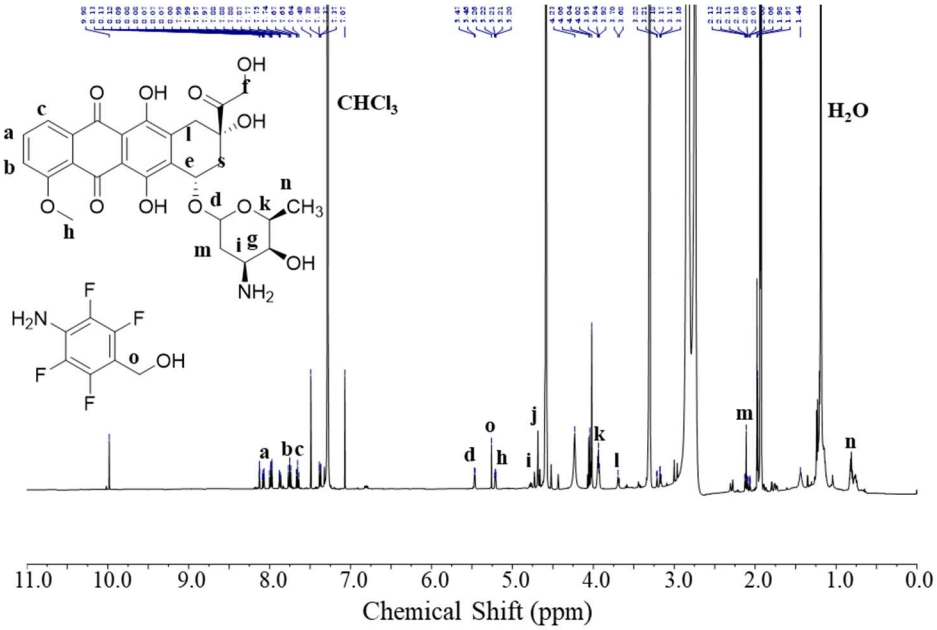


Fig. S33. ^{1}H NMR spectrum of the mixture of doxorubicin **15 and** compound **5** in $CDCl_3$ collected from a sample of irradiated prodrug **13** (60 Gy) by prep-HPLC (the two compounds were co-eluted, and could not be separated).

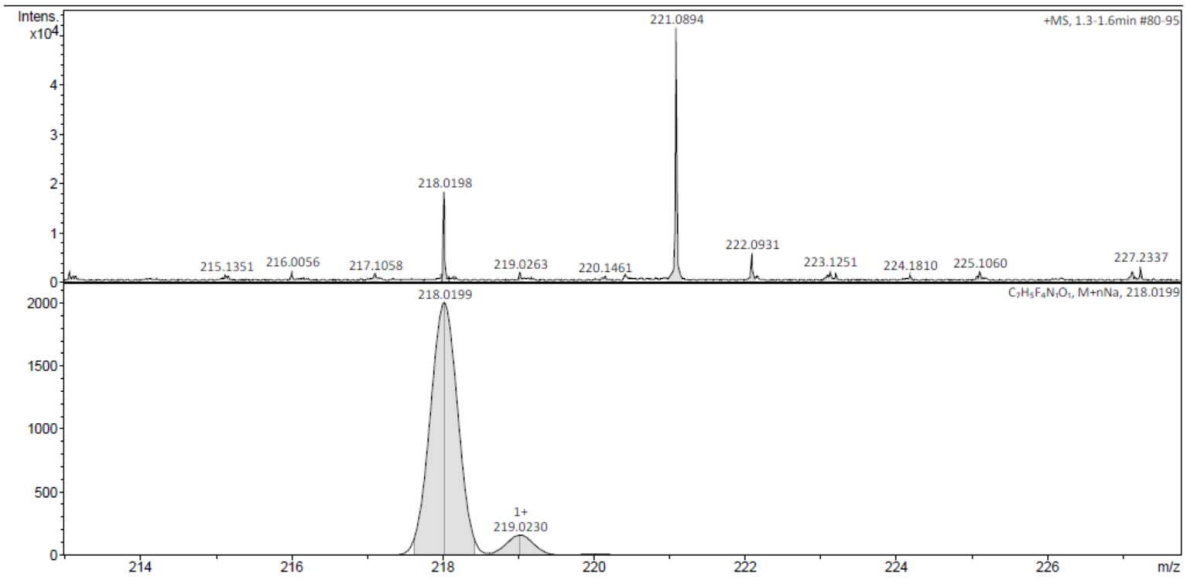


Fig. S34. HRMS spectrum of the mixture of compound **5** and doxorubicin **15** collected from the mixture of irradiated prodrug **13** (60 Gy) by prep-HPLC, showing the mass for compound **5**, (ESI) $C_7H_5F_4NO$ $[M+Na]^+$: *calcd.*: 218.0199; found: 218.0198.

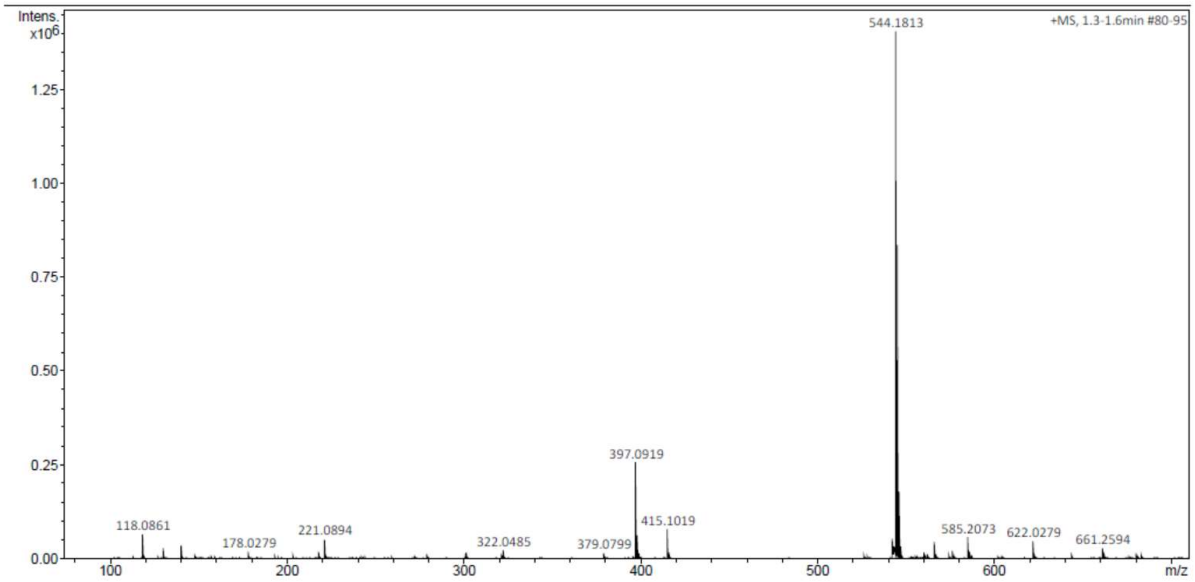


Fig. S35. HRMS spectrum of the mixture of compound **5** and doxorubicin **15** collected from the mixture of irradiated prodrug **13** (60 Gy) by prep-HPLC, showing the mass for doxorubicin **15**, (ESI) for $C_{27}H_{29}NO_{11}$ $[M+H]^+$: *calcd.*: 544.1813; found: 544.1813.

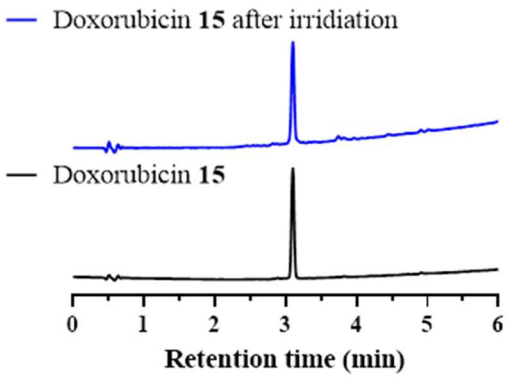


Fig. S36. HPLC traces (254 nm) of doxorubicin **15** before and after X-ray irradiation (60 Gy).

Cell viability against prodrug 13 before and after X-ray irradiation

HeLa cell viability was evaluated using an MTT assay as described above. The cells were treated with prodrug **13** (0.5, 1, 5 and 10 μM) for 4 h, and irradiated with X-ray at 0 Gy, 6 Gy, 12 Gy, 24 Gy, 36 Gy, 48 Gy and 60 Gy. Cells treated with 50% DMSO in DMEM were used as a negative control.

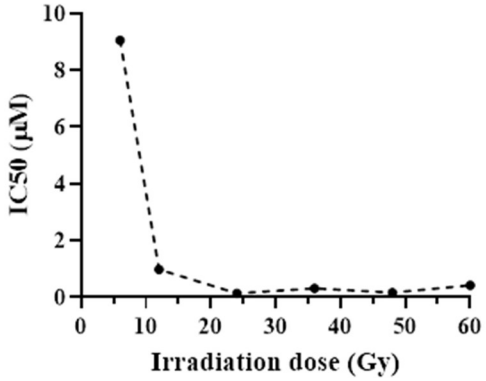


Fig. S37. Plot of IC50 values concluded from MTT assay of HeLa cells treated with of prodrug **13** with 6 to 60 Gy X-ray irradiation.

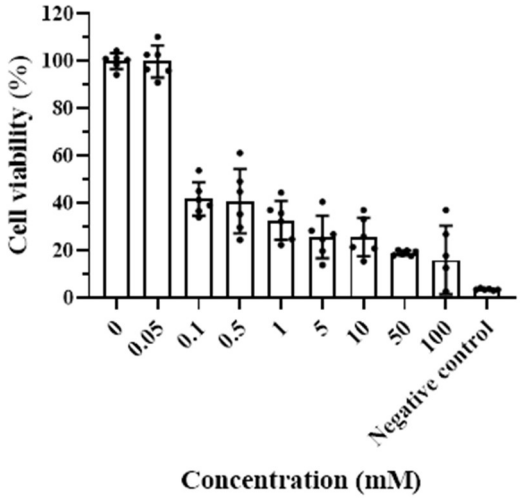


Fig. S38. Plot of IC50 values concluded from MTT assays of HeLa cells treated with prodrug **13** following 6 to 60 Gy X-ray irradiation. Data were presented as mean ± SD (n = 6 independent samples for each group). The experiments were repeated, independently, 5 times with similar results observed.

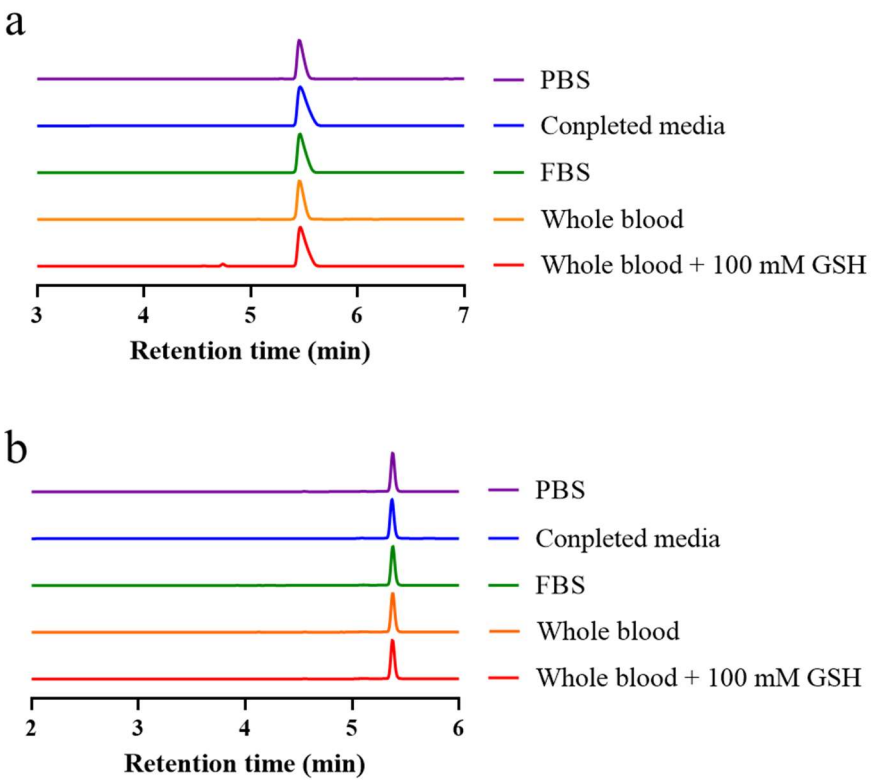


Fig. S39. Stability analysis of prodrugs (**a**) **11** and (**b**) **13** in PBS, complete media (containing 10 % fetal bovine serum in DMEM), fetal bovine serum (FBS), whole murine blood and whole

murine blood + 100 mM glutathione (GSH). Prodrugs (10 mM in DMSO) were diluted into the desired solutions to give a final concentration of 10 μM and incubated at room temperature for 72 h. The prodrugs were extracted from aqueous solutions using DCM, dried and redissolved in 1:1 (*v*/*v*) MeCN/water followed by HPLC analysis (254 nm). The experiments were repeated, independently, 3 times with similar results observed.

Tumour model development

To establish the HT-29 tumour model, HT-29 cells suspended in Matrigel were subcutaneously injected into the right flank subcutaneous tissues of the 6-8 week aged female BALB/c nude mice (1×10^6 cells/mouse). When the tumour size reached approximately 100 mm^3, the mice were randomly divided into 6 groups (n = 5) for the treatment with PBS, pazopanib **10**, prodrug **11**, PBS + X-ray, pazopanib 1**0** + X-ray and prodrug **11** + X-ray (doses for both pazopanib **10** and prodrug **11**: 100 mg/kg, intratumoural injections). X-ray irradiation was conducted 4 h after injection (6 Gy, 6 Gy/min). Tumour sizes of each group were measured every other day using a calliper. Tumour volume = (length) × $(width)^2/2$. Relative tumour volume was calculated as V/V_0 (V_0 was the initial tumour volume). The body weights of the mice were measured every 2 days. The endpoint criteria of the study were a tumour size greater than 2,000 mm^3 and weight loss exceeding 15% of the starting weight. Tumours of mice in different treatment groups were sectioned for H&E staining and Ki67 immunostaining analysis.

Doxorubicin **15**, prodrug **13**, PBS + X-ray, doxorubicin **15** + X-ray and prodrug **13** + X-ray (doses for both **15** and **13**: 10 mg/kg, intratumoural injections) were analysed in a similar manner. X-ray irradiation was conducted 4 h after injection (6 Gy, 6 Gy/min). Tumour sizes and body weights of mice were analysis as conducted above.

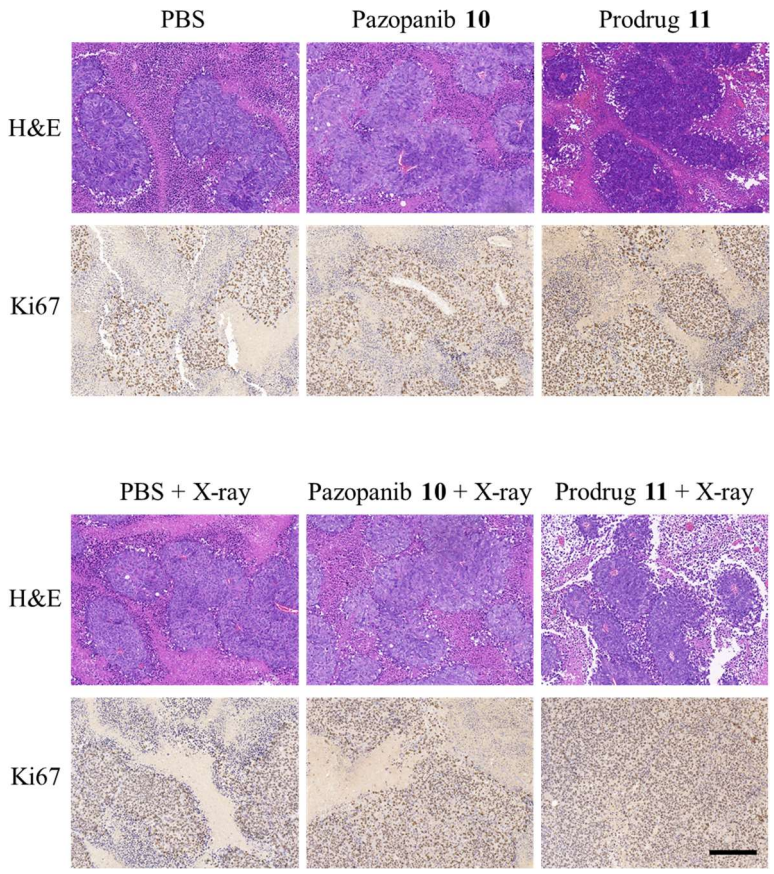


Fig. S40. Representative microscopic images of HT-29 tumour sections stained with H&E and Ki67 with Pazopanib and Prodrug **11**. Scale bar = 200 μm. The experiments were repeated, independently, 5 times with similar results observed.

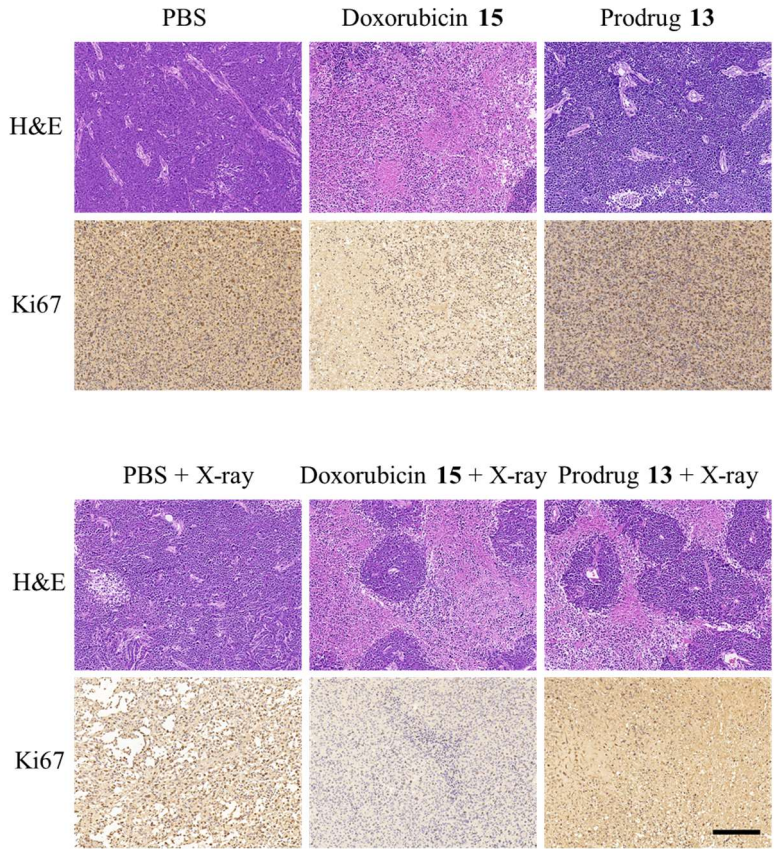


Fig. S41. Representative microscopic images of HeLa tumour sections stained with H&E and Ki67 with doxorubicin and prodrug **13**. Scale bar = 200 μm. The experiments were repeated, independently, 5 times with similar results observed.

Biosafety evaluations

Systemic toxicity was investigated by looking at the body weight changes, some biochemical markers in blood and histological changes to the main organs.

Hela tumour (around 100 mm^3) bearing female BALB/c nude mice (6-8 week aged) were randomly divided into 5 groups (n = 5) for the treatment with PBS, pazopanib **10**, prodrug **11**, doxorubicin **15** and prodrug **13** (pazopanib **10** and prodrug **11** dose: 100 mg/kg, doxorubicin **15** and prodrug **13** doses: 10 mg/kg, intratumoural injections). After 48 h, all mice were euthanized to collect blood and main organs for further tests. Blood samples were tested in terms of creatine kinase (CK), creatine kinase- muscle/brain (CK-MB), lactic acid

dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and alkaline phosphatase (ALP). Main organs were sectioned, attained with H&E and analysed immediately.

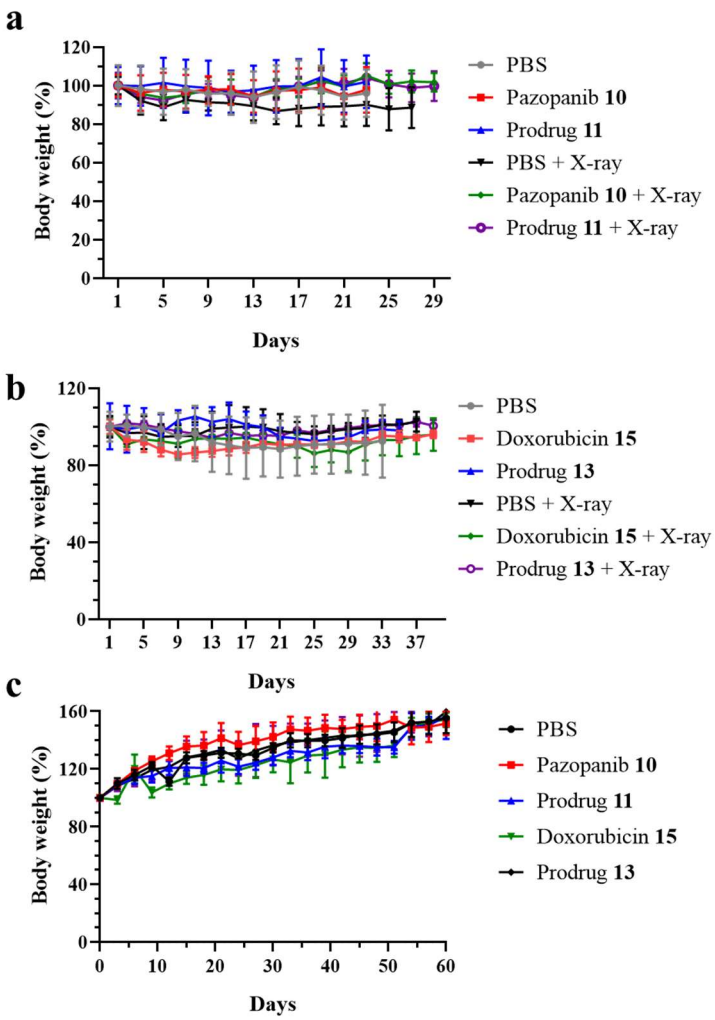


Fig. S42. Body weight changes in (**a**) HT-29 tumour bearing BALB/c nude mice treated with PBS, pazopanib **10** or prodrug **11** followed with or without 6 Gy X-ray irradiation. (**b**) HeLa tumour bearing BALB/c nude mice treated with PBS, doxorubicin **15** or prodrug **13** followed with or without 6 Gy X-ray irradiation. (**c**) Healthy BALB/c mice treated with PBS, pazopanib **10**, prodrug **11**, doxorubicin **15** and prodrug **13**. Data were presented as mean ± SD (n = 5 independent samples for each group).

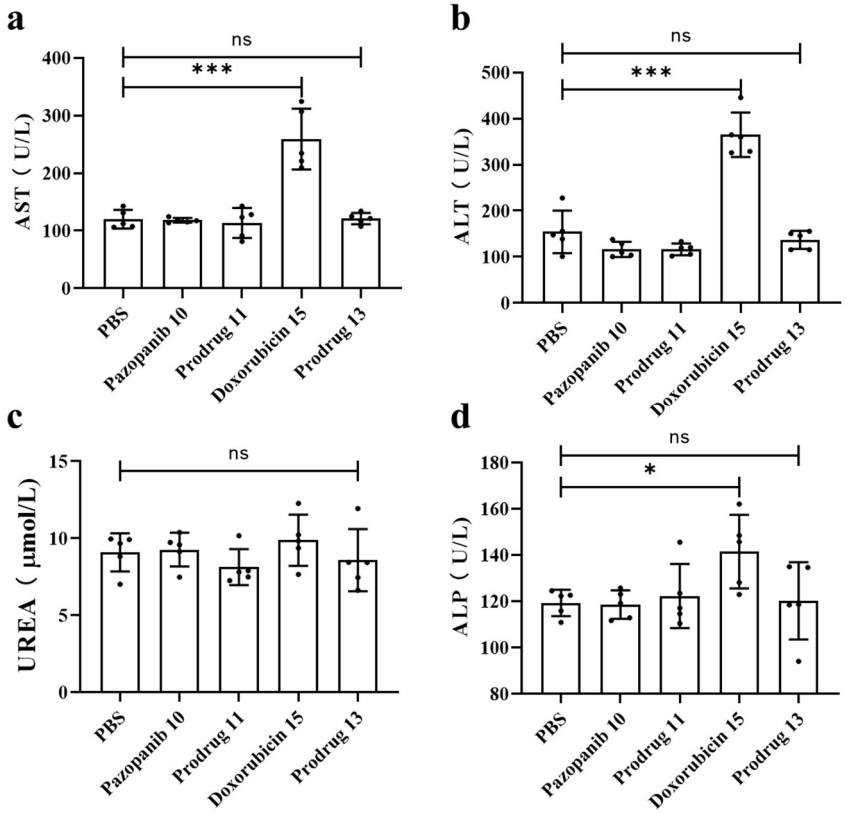


Fig. S43. Biochemical markers (**a**) AST, (**b**) ALT, (**c**) UREA and (**d**) ALP levels in plasma 48 h after treatments. Data were presented as mean ± SD (n = 5 independent samples for each group). Statistical analysis was performed using one-way ANOVA with Dunnett post-test compared to PBS treated mice, * $P = 0.045$, *** $P < 0.001$, ns (not significant).

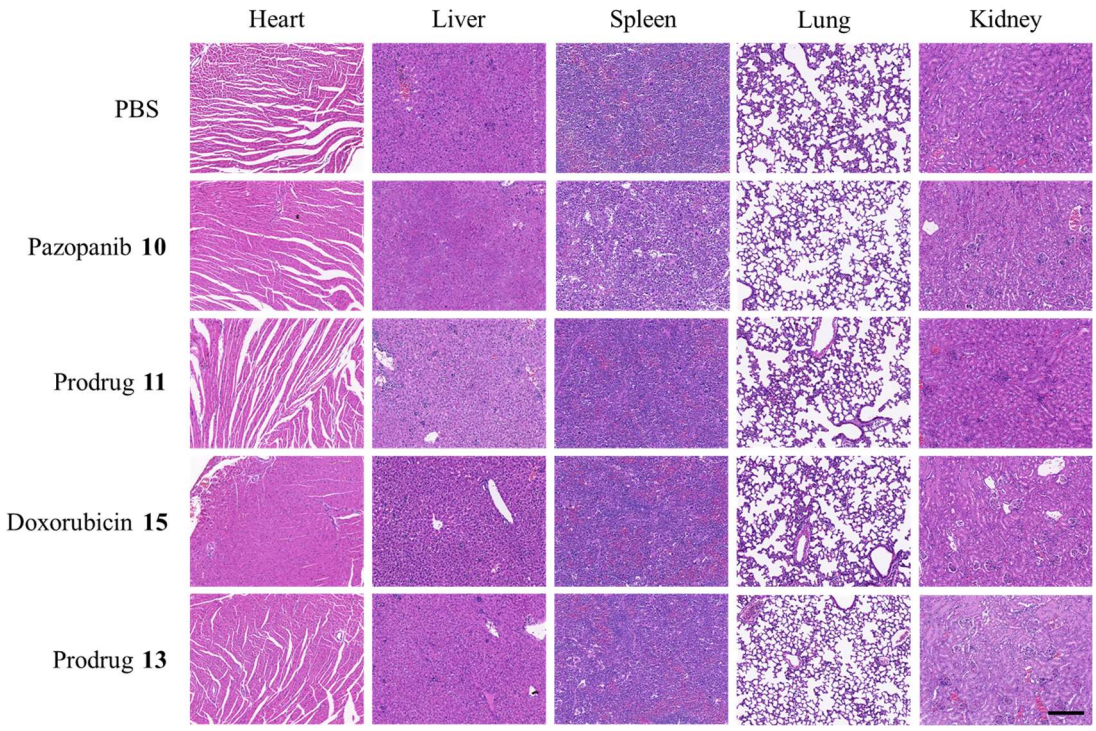


Fig. S44. Representative histopathological sections of organs collected from BALB/c mice 48 h after treatment. Free doxorubicin treated group experienced obvious pathological changes with atrophy and irregular arrangement of myocardial cells. The experiments were repeated, independently, 3 times with similar results observed.

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