Electronic Supplementary Information

for

A Small Molecule for TheraNOstic Targeting of Cancer Cells

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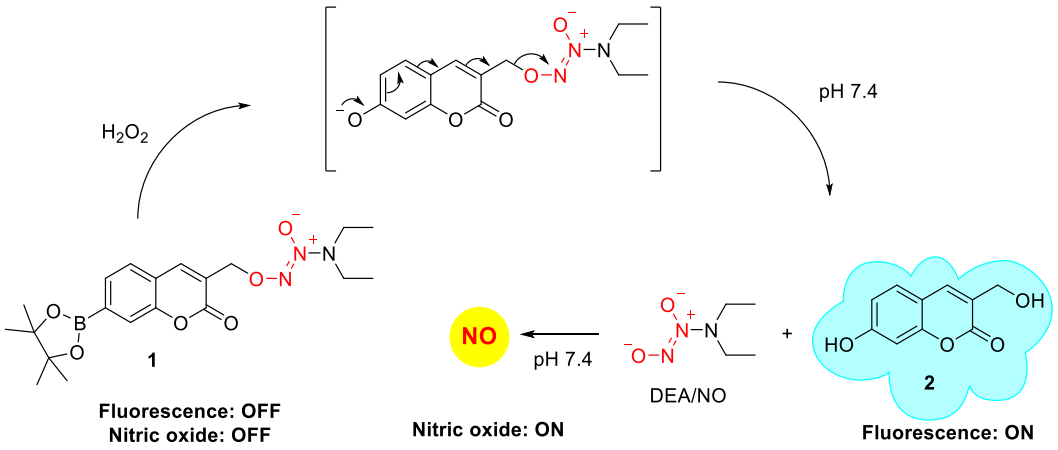
1. General

All reactions were conducted under nitrogen atmosphere. All the chemicals were purchased from commercial sources and used as received unless stated otherwise. Potassium Acetate (KOAc), [1,1′ Bis(diphenylphosphino)ferrocene]dichloropalladium(II), complex with dichloromethane ($Pdcl_2$(dppf)), N-Bromosuccinimide (NBS), azobisisobutyronitrile (AIBN), carbontetrachloride (CCl_4), N, N-Dimethylformamide (DMF), and tetrahydrofuran (THF), Sodium (Z)-1-(N,N-Diethylamino)diazen-1-ium-1,2-diolate (DEA/NO), for reaction were used as received, and petroleum ether and ethyl acetate (EtOAc) for chromatography were distilled before use. Column chromatography was performed on Rankem silica gel (60–120 mesh). ^{1}H and ^{13}C spectra were recorded on JEOL 400 MHz (or 100 MHz for ^{13}C) spectrometers using either residual solvent signals as an internal standard ($CHCl_3$ δ_H, 7.26 ppm, δ_C 77.2 ppm) or an internal tetramethylsilane (δ_H = 0.00, δ_C = 0.00). Chemical shifts (δ) are reported in ppm and coupling constants (*J*) in Hz. The following abbreviations are used: m (multiplet), s (singlet), d (doublet), t (triplet), dd (doublet of doublet). High-resolution mass spectra (HRMS) were obtained from HRMS-ESI-Q-Time of Flight LC/MS. FT-IR spectra were recorded using BRUKER-ALPHA FT-IR spectrometer. Melting points were measured using a VEEGO melting point apparatus in open glass capillary. Fluorescence and photometric measurements were performed using a Thermo Scientific Varioskan Flash microwell plate reader.

2. Synthesis and characterization: Compounds **2**[1] and **3**[2] were synthesized using a previously reported procedure and the analytical data that we collected were consistent with the reported values: ^{1}H-NMR spectra are included below.

(Z)-3,3-diethyl-1-((2-oxo-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-*2H*-chromen-3-yl)methoxy)triaz-1-ene 2-oxide (1): To a solution of compound **3** (200 mg, 0.70 mmol) in CCl_4 (5 mL), NBS (149 mg, 0.84 mmol) was added along with a trace amount of AIBN. The reaction mixture was refluxed for 4 h under nitrogen atmosphere. After cooling to room temperature, the solvent was removed under reduced pressure. The crude 3-(bromomethyl)-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2H-chromen-2-one was taken for next reaction without further purification. To a solution of **DEA/NO** (102 mg, 0.66 mmol) in THF (3 mL) under ice, 15-crown-5 (12 µL) was added and the mixture stirred at 0 ºC for 5 min under nitrogen atmosphere. A solution of 3-(bromomethyl)-7-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2H-chromen-2-one (crude, 200 mg, 0.55 mmol) in DMF (1 mL) was added to the reaction mixture at 0 ºC and stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure, diluted with 10 mL of water and the aqueous solution was extracted with EtOAc (3×5 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 (5 g), filtered and the filtrate was concentrated to give a crude compound. This crude was initially purified by silica gel column chromatography using EtOAc/pet ether (0 → 40 %) as the eluent. The resulting mixture was further purified using semi-preparative HPLC with C-18 semi-preparative column (9.4 mm × 250 mm, 5 µm; ZORBAX ODS), using a gradient of ACN and water (60 – 80 %), under ambient temperature with a flow rate of 2.5 mL/min to obtain **1** (12 mg, 5 %) as a semi solid; FT-IR (ν_{max}, cm^{-1}): 2923, 1723, 1550, 1508, 1359; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.75 (s, 2H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.46 (d, *J* = 7.7 Hz, 1H), 5.26 (d, *J* = 1.2 Hz, 2H), 3.17 (q, *J* = 7.1 Hz, 4H), 1.36 (s, 12H), 1.10 (t, *J* = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 160.1, 152.9, 139.6, 130.6, 127.3, 124.9, 122.7, 120.9, 84.6, 70.0, 48.6, 25.0, 11.7; HRMS (ESI) for $C_{20}H_{28}BN_3O_6$ $[M+Na]^+$: Calcd., 440.1968, Found, 440.1967.



Scheme S1. Proposed mechanism of activation of **1** by hydrogen peroxide to produce **2** and NO.

Nitric oxide detection from 1:[3] A 1 mM stock solution of compound **1** in DMSO and 10 mM stock solution of H_2O_2 in water were prepared. A typical reaction mixture consisted of compound **1** (25 µM) and H_2O_2 (250 µM, 10 eq.,) was prepared by mixing 12.5 µL of **1** and 12.5 µL of H_2O_2 from the stock solutions with 475 µL of pH 7.4 phosphate buffer (10 mM) at 37 ºC. An aliquot of the reaction mixture (10 µL) from the reaction vial was injected into a Sievers Nitric Oxide Analyzer (NOA 280i) using argon as the carrier gas. The amount of NO released was estimated using a standard calibration curve generated using sodium nitrite $NaNO_2$ solution of concentration from 0 – 50 µM using NOA Analyzer (Y = 140.2X; R^2 = 0.996). The data represented here is average of 3 repeats.

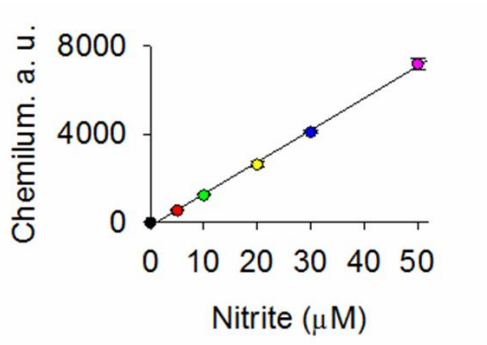


Figure S1. Calibration curve for nitrite in pH 7.4 phosphate buffer at 37 °C.

Fluorescence measurement from 1: A 1 mM stock solution of compound **1** in DMSO and a 10 mM stock solution of H_2O_2 in water were prepared. The experiment was performed in 96 well plate. In the control experiment, 2.5 µL of compound (25 µM) and 97.5 µL of phosphate buffer pH 7.4. In another set serving as

H_2O_2 activated set 2.5 µL of compound **1** (25 µM) was added to 2.5 µL of H_2O_2 (final concentration of **1** was 25 µM in both blank and reaction; H_2O_2 used was 250 µM). The amount of **2** released was estimated using a standard calibration curve generated using authentic **2** solution of concentration varying from 0 – 25 µM (Y = 21.8X; R^2 = 0.994). Fluorescence was measured using a Thermo Scientific Varioskan Flash microwell plate reader (excitation 315 nm; emission 460 nm). The data represented here is average of 3 repeats.

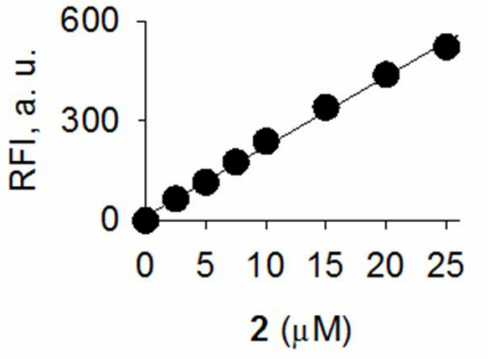


Figure S2. Calibration curve for compound **2** in pH 7.4 phosphate buffer at 37 °C.

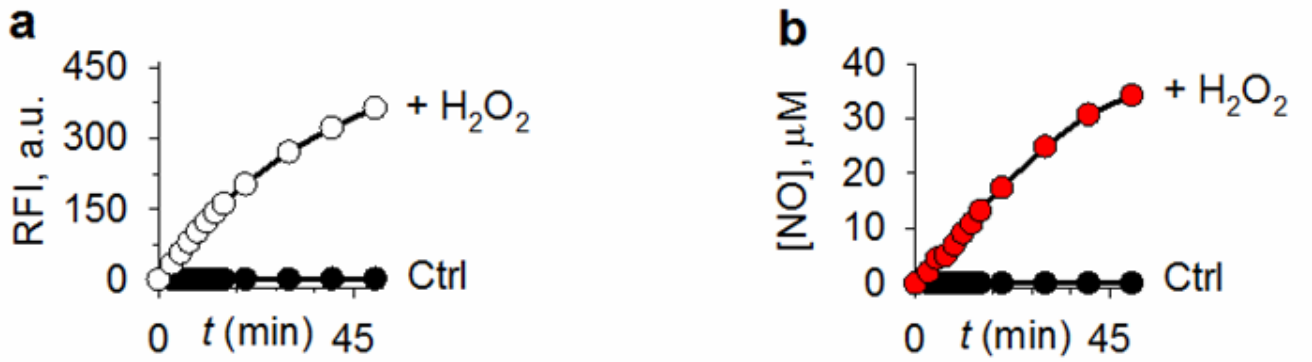


Figure S3. (a) Time course of enhancement of fluorescence signal (excitation 315 nm, emission 460 nm) attributable to **2** upon incubation of **1** (25 µM) with and without H_2O_2 (10 eq). (b) Time course of enhancement of NO upon incubation of **1** (25 µM) with and without H_2O_2 (10 eq).

Selectivity studies of 1 with oxidants and reductants: A 1 mM stock solution of **1** in DMSO and 10 mM stock solution of various reactive species were prepared. A reaction mixture of **1** (10 µM, 1 eq.,) and various analytes (100 µM, 10 eq.,) were prepared by mixing 1 µL of **1** and 10 µL of analytes from the stock solutions to 89 µL of pH 7.4 phosphate buffer (10 mM) at 37 ºC. Fluorescence was measured using a Thermo Scientific

Varioskan Flash microwell plate reader (excitation 315 nm; emission 460 nm). The data represented here is average of 3 repeats.

Fluorescence emission from 1 with different equivalents of H_2O_2: A 1 mM stock solution of **1** in DMSO and 10 mM stock solution of H_2O_2 in water were prepared. A reaction mixture of **1** (25 µM, 1 eq.,) and H_2O_2 (0 - 500 µM, 0 - 20 eq.,) were prepared by mixing 2.5 µL of **1** and respective volume of H_2O_2 from the stock solutions to pH 7.4 phosphate buffer (10 mM, final volume 100 µL) at 37 ºC. Fluorescence was measured using a Thermo Scientific Varioskan Flash microwell plate reader (excitation 315 nm; emission 460 nm). The data represented here is average of 3 repeats.

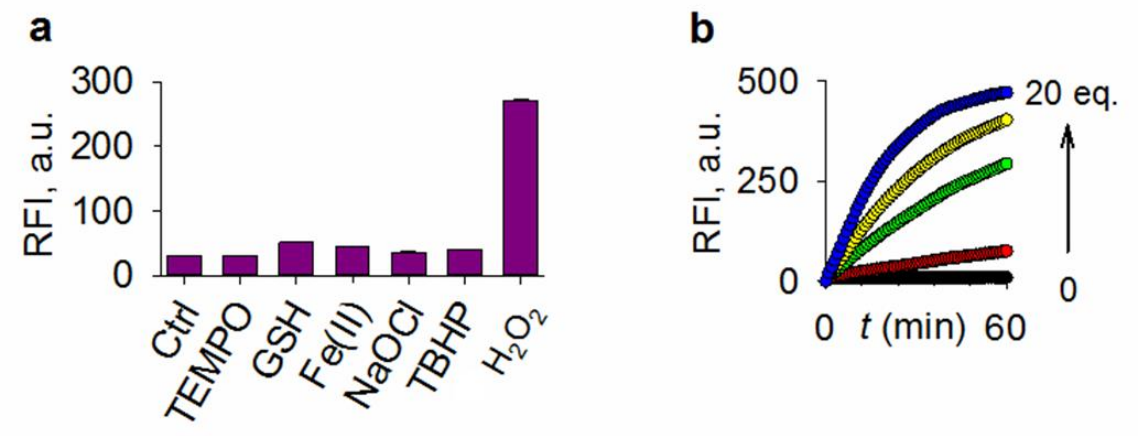


Figure S4. (a) Fluorescence response of **1** (10 µM) with various reactive species (100 µM) at 37 °C in pH 7.4 buffer after 30 min incubation. Ctrl: buffer; TEMPO: 2,2,6,6-tetramethylpiperidinyloxy; GSH: glutathione; Fe(II): $FeCl_2$; NaOCl: sodium hypochlorite; TBHP: *tert*-butyl hydroperoxide; H_2O_2. (b) Time course of fluorescence measurements during incubation of **1** (25 µM) in the presence of H_2O_2 at various concentrations.

Fluorescence properties of compound 2: A 1 mM stock solution of compound **2** in DMSO and a 100 mM stock solution of H_2O_2 in water were prepared. The experiment was performed in 96 well plates. In the control experiment, 2.5 µL of compound **2** (25 µM) was added to 97.5 µL of phosphate buffer pH 7.4. In another set serving as H_2O_2 activated set 2.5 µL of compound **2** (25 µM) was added to 0.25 µL and 0.75 uL of 100 mM H_2O_2 (For 10 and 30 eq., H_2O_2 respectively). Fluorescence was measured after 30 min using a Thermo Scientific Varioskan Flash microwell plate reader (excitation 315 nm; emission 460 nm). The data represented here is average of 3 repeats.

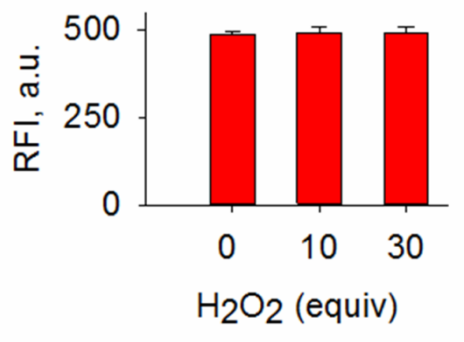


Figure S5. Fluorescence properties of compound **2** with different equivalents of H_2O_2 in pH 7.4 phosphate buffer at 37 °C.

Fluorescence emission and nitrite release from 1 in HeLa cells: Cells were suspended in HBSS medium and plated in a 96-well plate ($2.5x10^4$ cells/200 µL). A 10 mM stock solution of **1** in DMSO and 10 mM stock solution of H_2O_2 in water were prepared. In control experiment, compound **1** (25 µM) was added to the cell suspension. In another set serving as H_2O_2 activated set, cells were pre-treated with H_2O_2 (100 µM) for 30 min, followed by compound **1** was incubated for 30 min at 37 ºC and fluorescence emission was measured using a Thermo Scientific Varioskan Flash microwell plate reader (excitation 315 nm; emission 460 nm). For nitrite measurement, Griess' reagent (14 µL for 200 µL reaction mixture, Sigma Aldrich) was added, and incubated at 37 ºC for 25 min before measuring OD at 535 nm using a Thermo Scientific Varioskan Flash microwell plate reader. The amount of nitrite release was estimated using a standard calibration curve generated using sodium nitrite $NaNO_2$ solution of concentration from 0 – 50 µM (Y = 0.0145 X; R^2 = 0.998). The data represented here is average of 3 repeats.

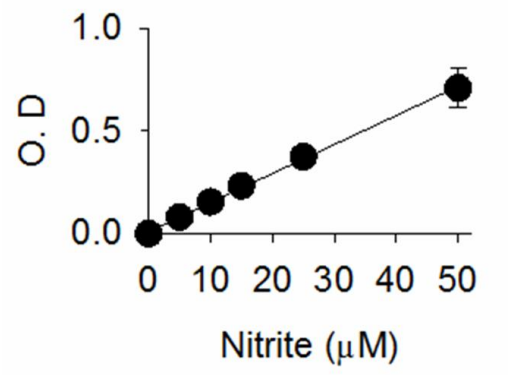


Figure S6. Calibration curve for nitrite in HBSS Buffer at 37 °C. (O.D = Optical Density).

Fluorescence image of 1 activated by exogenous H_2O_2: HeLa cells (50000) were seeded in 35 mm glass bottom dishes in DMEM media supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic solution and grown overnight in an atmosphere of 5% CO_2 at 37 °C. The cells were washed with PBS (1X) and treated with compound **1** (25 µM) in DMEM and incubated for 30 minutes. The cells were washed with PBS, followed by treatment with H_2O_2 (100, 200 µM) in DMEM for additional 30 minutes, followed by imaging in live cell imaging solution (Invitrogen A14291DJ) using Olympus IX83 fluorescence microscope. The images was acquired and analysed using Slidebook 6.0 software (3i, Intelligent Imaging Innovations, Denver, CO) at identical exposure and camera settings.

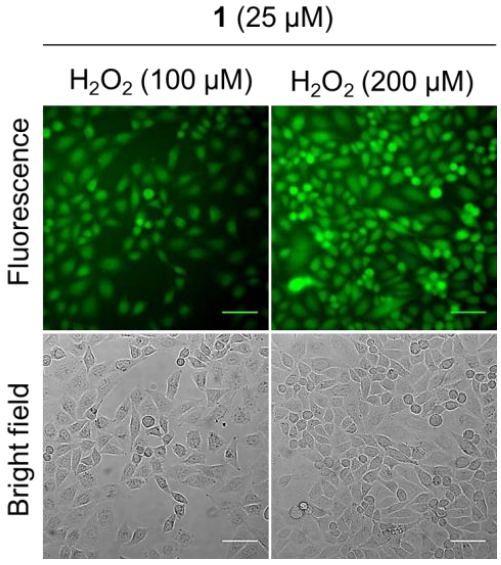


Figure S7. Fluorescence images of HeLa cells incubated with **1** (25 µM) with increasing concentration of H_2O_2 (100 and 200 µM). Images were taken using DAPI channel, pseudocolor (green) was given for better visualisation. Scale bar: 50 µm.

FACS analysis of HeLa cells with 1: HeLa cells were seeded at 1×10^5 cells/well in 6 well plate for overnight in DMEM media supplemented with 10% FBS and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 °C. After incubation, old media was removed and the cells were washed with 1 mL of PBS (1X) buffer. Then 1 mL of fresh DMEM media was added along with compound **1** (25 µM) and cells were incubated for 30 minutes at 37 °C. After 30 minutes, media was removed, cells were washed with 1 mL of PBS (1X). 1 mL of fresh DMEM media was added along with H_2O_2 (100, 250 µM) and cells were incubated for 30 minutes. After 30 minutes, media was removed, cells were washed with 1 mL of PBS (1X) and cells were detached by

trypsination. Cells were centrifuged at 1000 rpm, 22 °C for 5 min. Supernatant were removed and 500 µL of PBS (1X) was added to the cell pellet. Samples were illuminated with a UV laser at 355 nm on a Flowcytometry (BD LSRFortessa SORP cell analyser). The data represented here is average of 2 repeats.

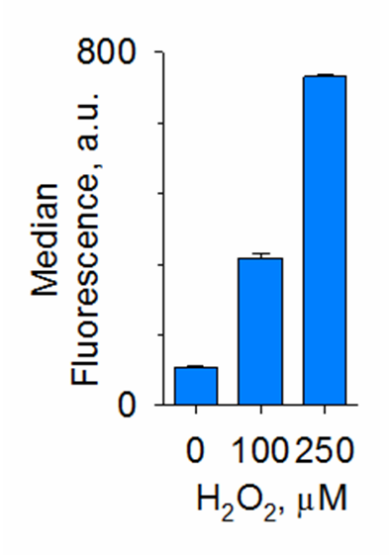


Figure S8. Flow cytometry analysis of HeLa cells incubated with **1** at 25 µM with increasing concentration of H_2O_2 (excitation 355 nm, emission 460 nm).

Confocal Image of HeLa cells with 1: HeLa cells were seeded at 0.5×10^5 cells/well in 4-well chamber for overnight in DMEM medium supplemented with 10% FBS and 1% antibiotic solution in an atmosphere of 5% CO_2 at 37 °C. After incubation, old media was removed and the cells were washed with 500 µL of PBS (1X) buffer. Then 500 µL of fresh DMEM media was added along with compound **1** (25 µM) and cells were incubated for 30 minutes at 37 °C. After 30 minutes, media was removed, cells were washed with 200 µL of PBS (1X). Then 500 µL of fresh DMEM media was added along with H_2O_2 (100 µM) and cells were incubated for 30 minutes. After 30 minutes, media was removed, cells were washed twice with 200 µL of PBS (1X) followed by cells were stained with LysoTracker for 5 min and imaged on a Zeiss LSM 710 confocal microscopy with 405 nm, 488 nm laser lines at 2% power using a 63X oil immersion objective (DAPI channel was used for **1** and LysoTracker Green DND-26 channel were used for LysoTracker). Images were analysed by ImageJ software. DAPI channel was used for **1** and pseudocolor (red) was given for the better visualisation.

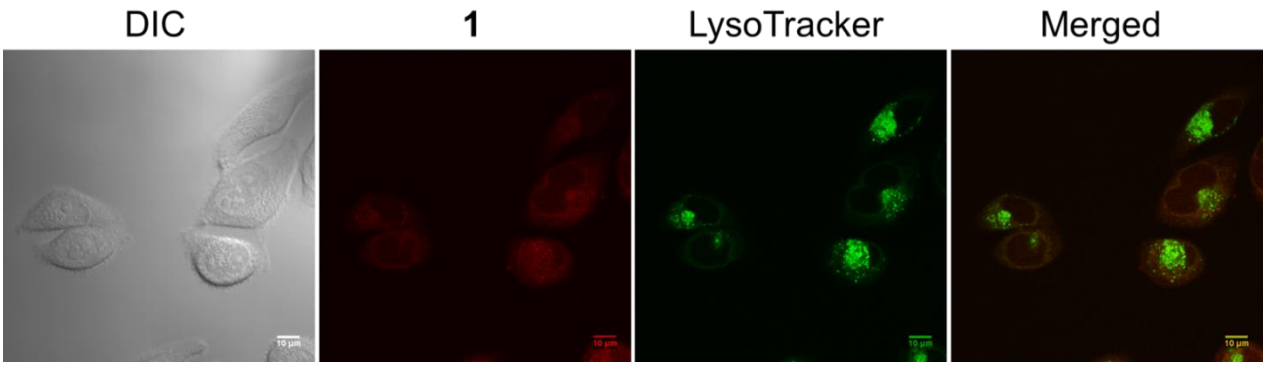


Figure S9. Subcellular localization of **1** in HeLa cells. Cells were treated with **1** (25 μM) for 30 min. Then, cells were treated with 100 μM H_2O_2 and further incubated for 30 min. Lysosomal localization was identified by LysoTracker-green. The excitation channel for **1** and LysoTracker were 405 and 488 nm, respectively. Scale bar: 10 μm.

cGMP measurement. HEK 293T cells were maintained in DMEM with foetal calf serum (10 %), penicillin (100 mg L^{-1}) and streptomycin (100 mg L^{-1}) at 37 °C in a 5 % CO_2 humidified incubator. HEK 293T cells were transfected with pGFP2-GAFa-Rluc[4] in a 12-well plate using Turbofect (Thermo Fisher Scientific) according to the manufacturer's protocol. After 48 h, cells were harvested, and reseeded (10^5 cells per well) in a 96-well plate in Live Cell Imaging Solution (Invitrogen) and treated with various compounds (as indicated). After 10 min, DeepBlueC (5 μM, ThermoFisher, USA) was added and BRET (Bioluminescence Resonance Energy Transfer) measurements were made in an Infinite M1000 PRO plate reader (Tecan, Austria) at the channel of BLUEI (luminescence) and Green1 (fluorescence). The data represented here is average of 3 repeats and statistical analysis was done using t-test.

Cell culturing and transfection. All cell lines were cultured in the modified Dulbecco's medium (DMEM) containing 3.7 g/l sodium bicarbonate, 110 mg sodium pyruvate, antibiotics (100 units/ml penicillin-streptomycin) and 10% FBS. Cells were incubated at 37 °C in a humidified incubator in presence of 5% CO_2 and 20% oxygen for all experiments.

Stable cell line generation. For gene expression knockdown, plasmids encoding gene specific shRNAs (targeting catalase gene or containing the scrambled shRNA), from Broad Institute's TRC shRNA library

(Sigma, USA) were used to generate the stable HeLa cell lines, which were selected on puromycin (3 µg/ml) for 48 hours before experiments.

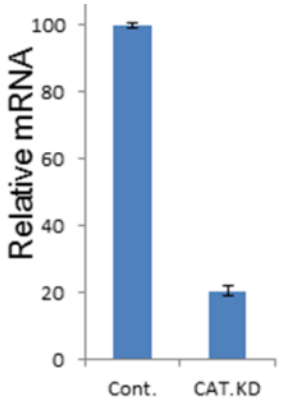


Figure S10. Relative mRNA level.

Measurements of ROS with H_2DCFDA dye. ROS detection was performed using M1000 multimode fluorescence plate reader (Tecan, Austria). Briefly, the cells were washed with PBS (1X) and incubated with 10 µM H_2DCFDA (2′,7′-Dichlorofluorescin diacetate) for 30 minutes in dark. Subsequently the cells were washed and fluorescence was detected at excitation and emission wavelength as 495 nm and 525 nm respectively. The fluorescence value recorded was normalized to 10^3 cells.

Real-time quantitative PCR. Total RNA was isolated from treated and untreated cells using the RNA isolation kit (RBC Bioscience Inc., UK). The cDNA was synthesized using random hexamers and RevertAid Reverse Transcriptase enzyme. Quantitative real time PCR was done using Power SYBR green master mix using Rotogene-Q real time cycler according to manufacturer's instructions. The β-actin expression was used as an internal control for expression normalization. Primers used for analysis are listed below.

Gene Name	Primers
β -actin	Forward 5′CCAACCGCGAGAAGATGAC 3′ Reverse 5′CAGAGGCGTACAGGGATAGC 3’
Catalase	Forward 5’ TGGGATCTCGTTGGAAATAACAC3’ Reverse 5’ TCAGGACGTAGGCTCCAGAAG3’

Fluorescence response from 1 with WT and KD cells: 2 x 10^4 cells were seeded in 24 well plate and incubated overnight. Cells were washed with PBS (1X) and treated with **1** (100 µM) in live cell imaging buffer at 37 °C for 30 minutes in dark. Subsequently the cells were washed with PBS (1X) and fluorescence was recorded using an excitation and emission wavelength of 315 nm and 460 nm respectively an Infinite M1000 PRO plate reader (Tecan, Austria). The data represented here is average of 3 repeats and statistical analysis was done using t-test.

Fluorescence imaging of 1 activated by endogenous H_2O_2: Four different cell lines, HeLa, A549, MRC5 and MDA-MB-231 were seeded overnight at a density of 1×10^5 cells in 35 mm glass bottom dishes in DMEM supplemented with 10% FBS and 1% antibiotic solution at 5% CO_2 and 37 °C. The cells were washed with PBS (1X) and treated with with compound **1** (25 µM) in DMEM and incubated for 6 h. The cells were washed with PBS, followed by imaging in live cell imaging solution (Invitrogen A14291DJ) using Olympus IX83 fluorescence microscope. The images was acquired and analysed using Slidebook 6.0 software (3i, Intelligent Imaging Innovations, Denver, CO) at identical exposure and camera settings. All the images were acquired at identical imaging conditions and acquisition parameters. Briefly, the intensity measurements were made using ImageJ on the acquired images. For this, individual cells were selected on the image and total intensity was determined and an average for all the regions was taken from one image for analysis. Data from atleast 5 – 10 regions per dish were used.

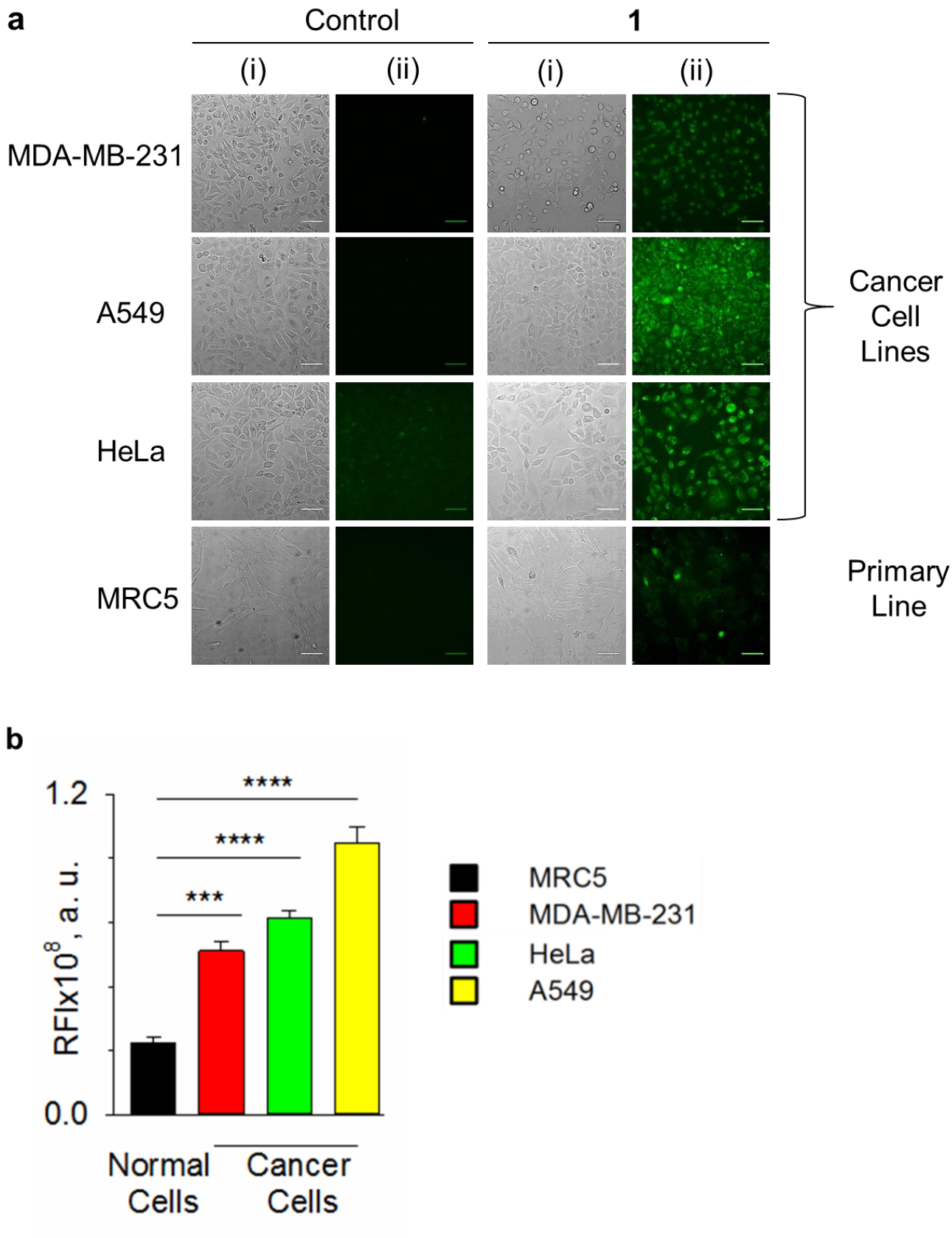


Figure S11. (a) Fluorescence images of different cells treated with **1** (25 μM) after 6 h at 37 °C. Images were taken using DAPI channel and pseudocolored (in green) for better visualisation. (i) Bright field image, (ii) Fluorescence image. Scale bar = 100 μm. All images were acquired with identical parameters (gain 3, intensification 200 and ND:75). (b) Quantification of fluorescence intensity was done using ImageJ software and statistical analysis was done using t-test. ***p-value 0.0002, ****p-value < 0.0001.

53BP1 (p53 Binding Protein 1) foci formation assay: HeLa cells stably expressing GFP-53BP1 were used for live cell imaging experiments.[5] The cells were treated with different concentrations of **1** for 6h before imaging. For imaging, the cells were seeded in glass bottom dishes and treated with indicated compounds and imaged in an IX83 inverted fluorescence microscope (Olympus) using 60X objective and data was analysed using Slidebook 6.0 software (Intelligent Imaging Innovations, Denver, CO). Images were taken in the GFP channel with fixed acquisition settings.

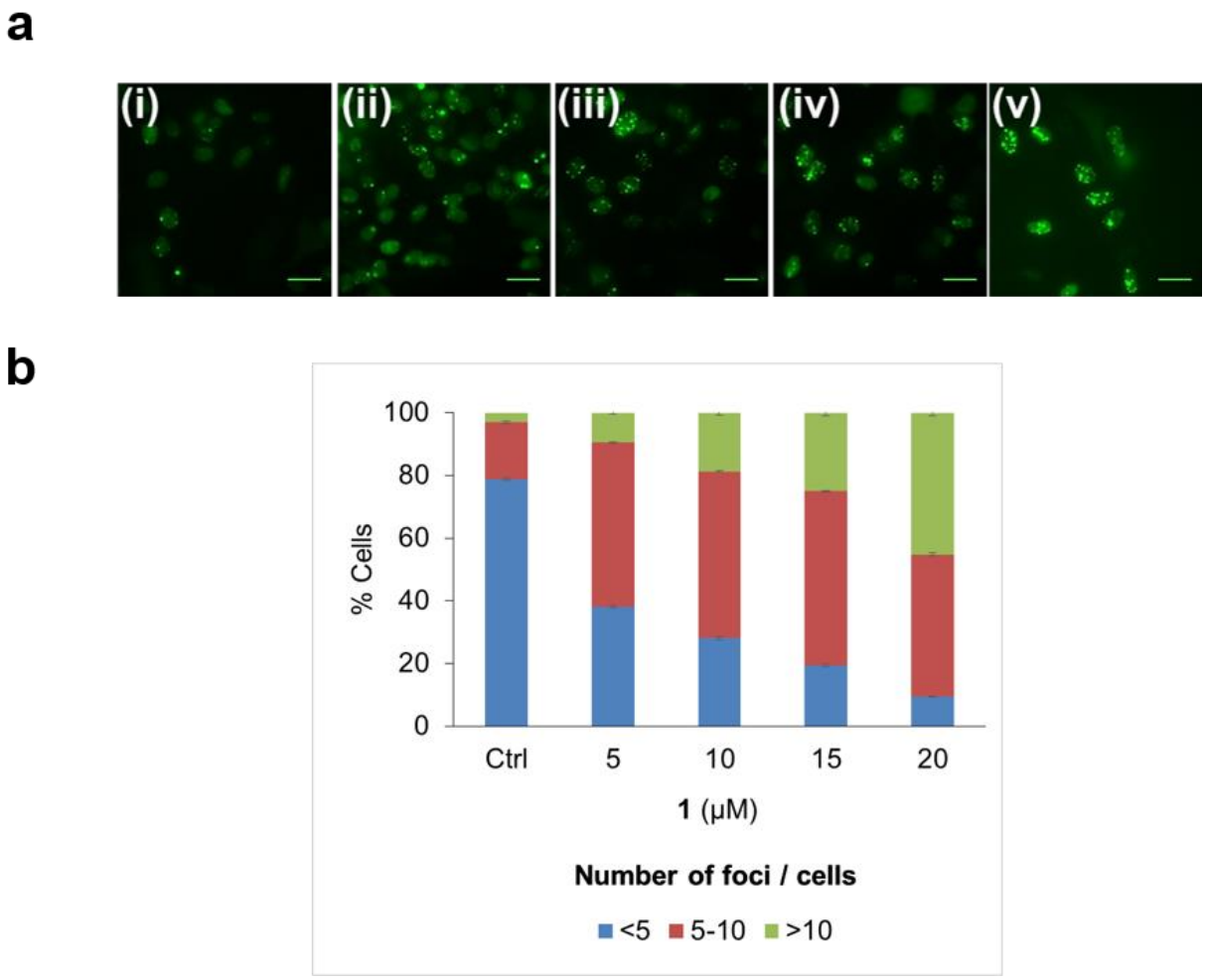


Figure S12. (a). Formation of 53BP1 foci upon exposure of (i) Control, (ii) 5 μM of **1**, (iii) 10 μM of **1**, (iv) 15 μM of **1**, (v) 20 μM of **1**. Images were taken in GFP channel. (b) Quantitation of 53BP1-GFP foci: Number of foci per cell were counted and the cells were classified in three categories (<5; 5 – 10; >10). Scale bar = 100 μm. All images were acquired with identical parameters (Camera gain 3, intensification 200 and ND: 75).

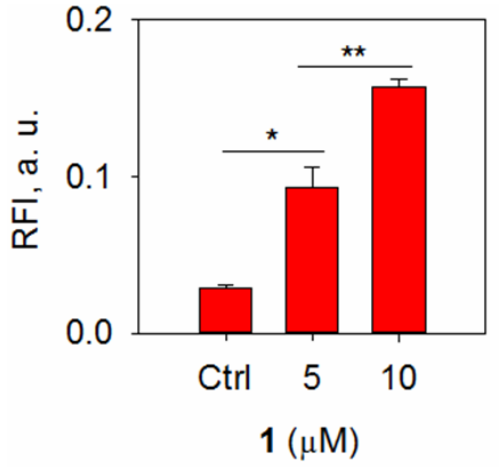


Figure S13. Quantification of fluorescence intensity attributable to the formation of **2** was done in the DAPI channel using ImageJ software and statistical analysis was done using t-test. *p-value 0.0112, **p-value 0.0086.

Cytotoxicity assay with normal vs cancer cells: Cytotoxicity assay was done using Alamar Blue cell viability assay. Briefly, 2 x 10^4 cells were seeded in 24 well plate and incubated overnight. Cells were washed with PBS (1X) and treated with **1** (10 and 25 µM) in media and incubated at 37 °C for 12 hours in dark. Subsequently the cells washed with PBS (1X) and Alamar Blue dye was added to each well as final concentration of 100 µM in media and incubated for 4 hours. Fluorescence was recorded using an excitation and emission wavelength of 590 nm and 600 nm respectively using an Infinite M1000 PRO plate reader (Tecan, Austria). The data represented here is average of four repeats and statistical analysis was done using student's t-test.

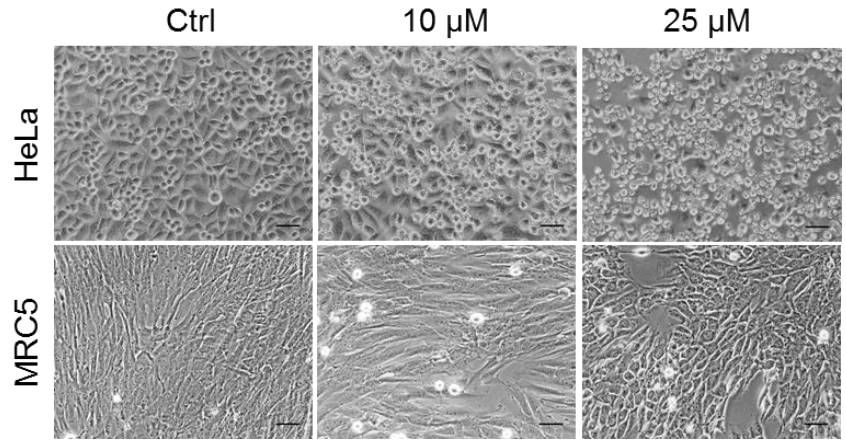
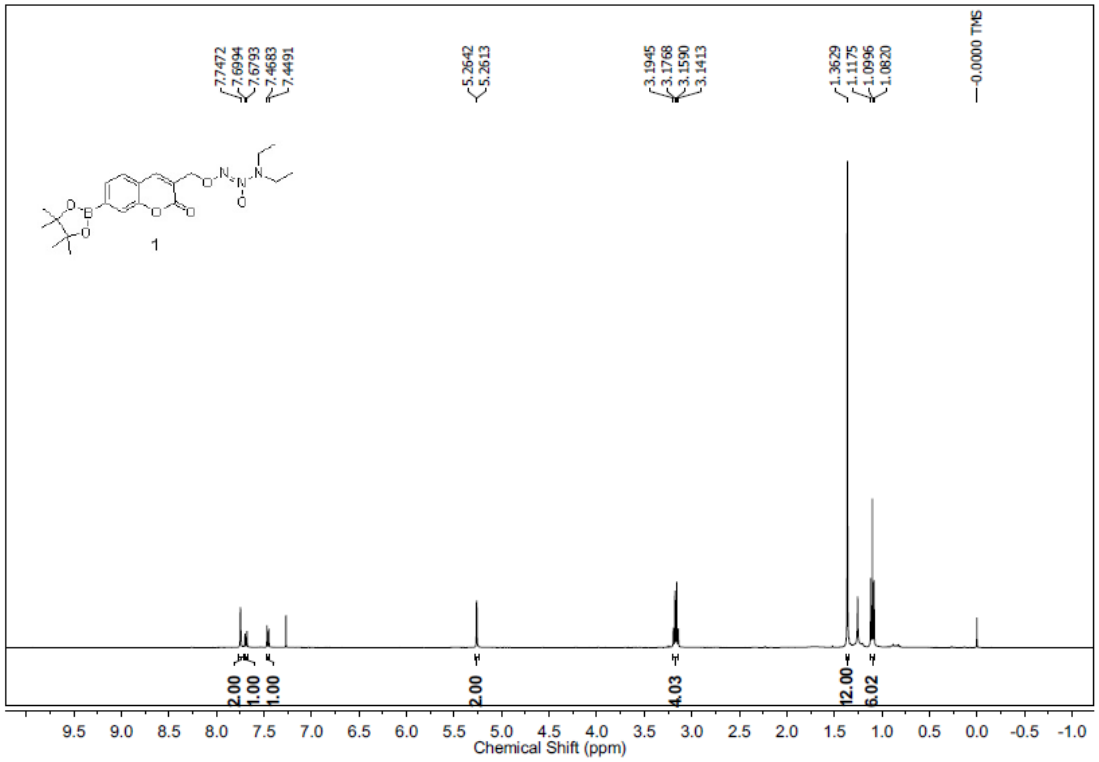
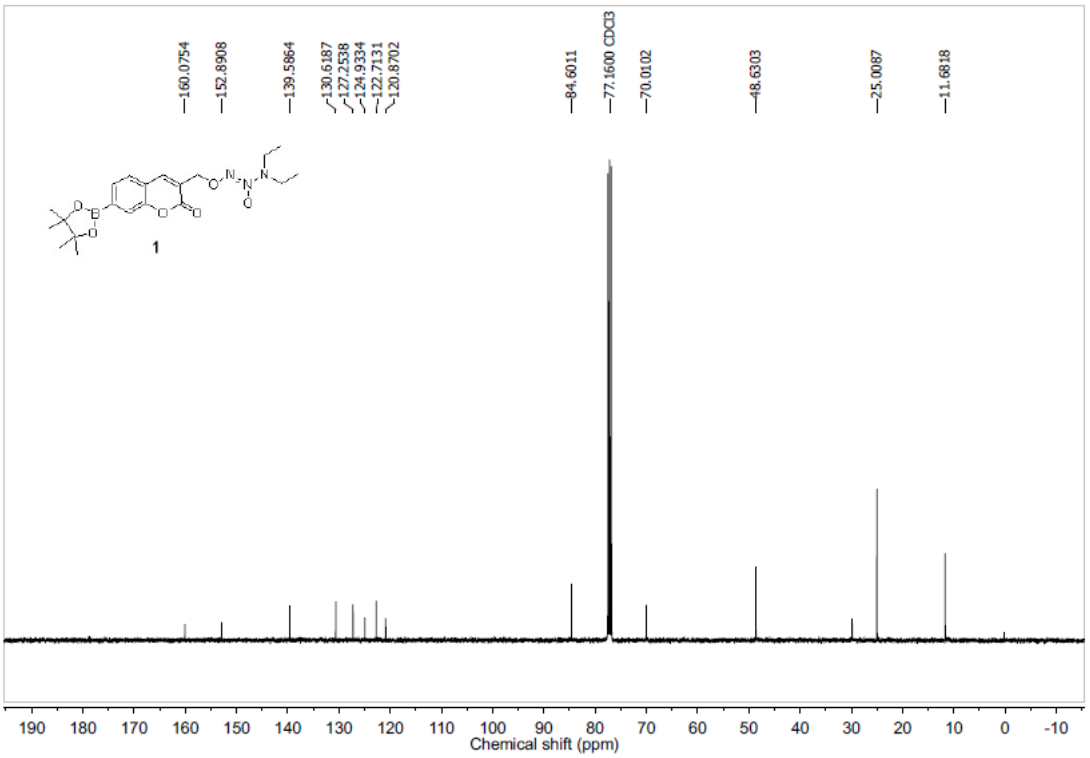


Figure S14: The cytotoxic effect of **1** on HeLa (cancer cells) and MRC5 (normal fibroblast). Scale bar = 50 µm.

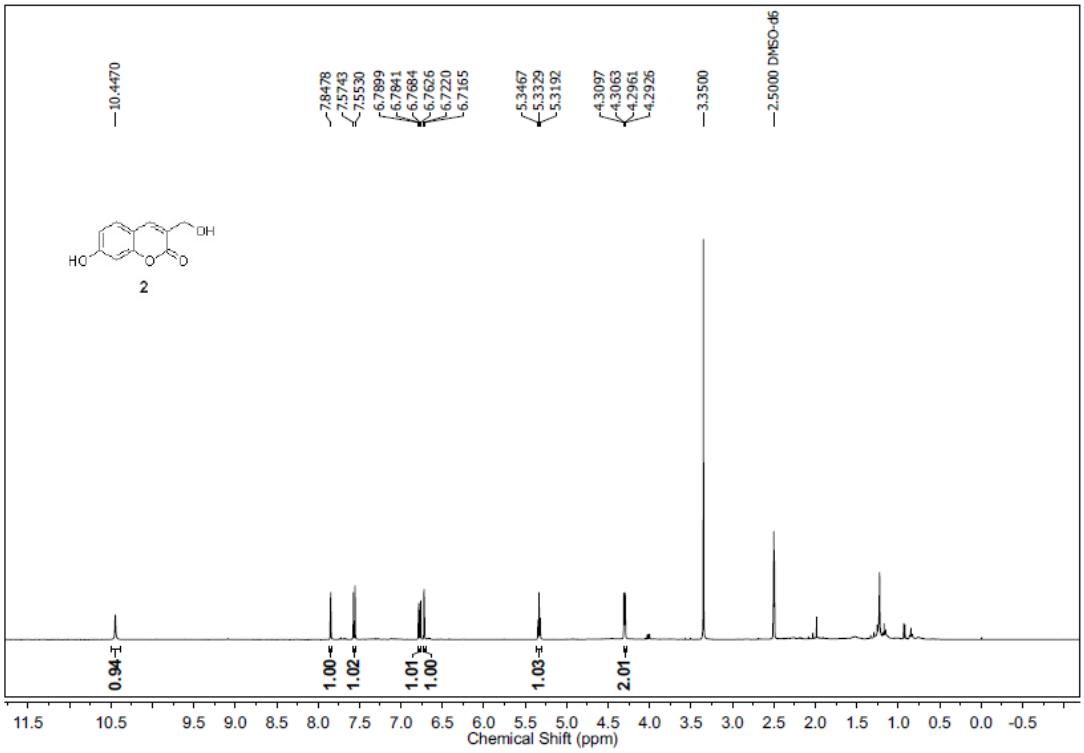
^{1}H-NMR Spectrum for Compound 1:



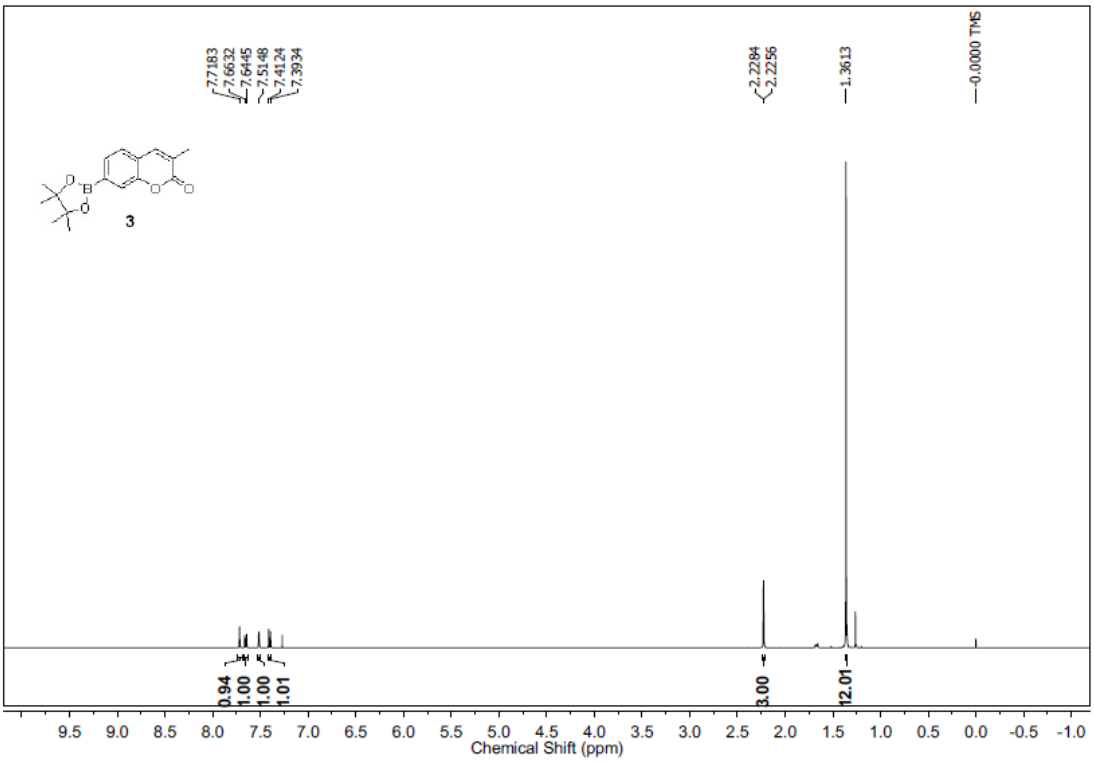
^{13}C-NMR Spectrum for Compound 1:



^{1}H-NMR Spectrum for Compound 2:



^{1}H-NMR Spectrum for Compound 3:



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