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Electronic Supplementary Information

Proof-of-Principle for Two-Stage Photodynamic Therapy: Hypoxia Triggered Release of Singlet Oxygen

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1. General Information

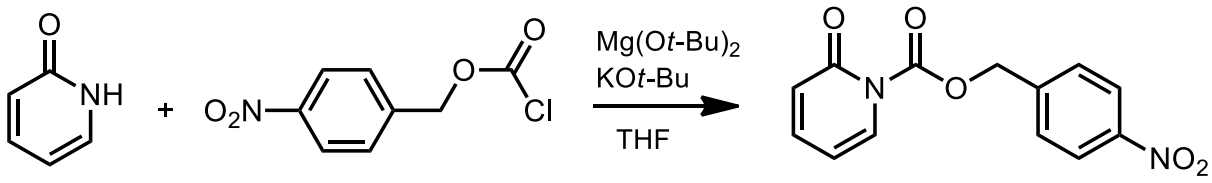
All reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions were monitored by thin layer chromatography using Merck TLC Silica gel 60 F254. Column chromatography was performed by using Merck Silica Gel 60 (particle size: 0.040-0.063 mm, 230-400 mesh ASTM).

The ^{1}H and ^{13}C NMR spectra were recorded using Bruker DPX-400 (operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) at 298 K using deuterated solvents with tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in parts per million (ppm) and coupling constants (*J* values) are given in Hz. Splitting patterns are indicated as follow s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded with Agilent Technologies 6224 TOF LC/MS.

2. Experimental Procedures and Characterization Data

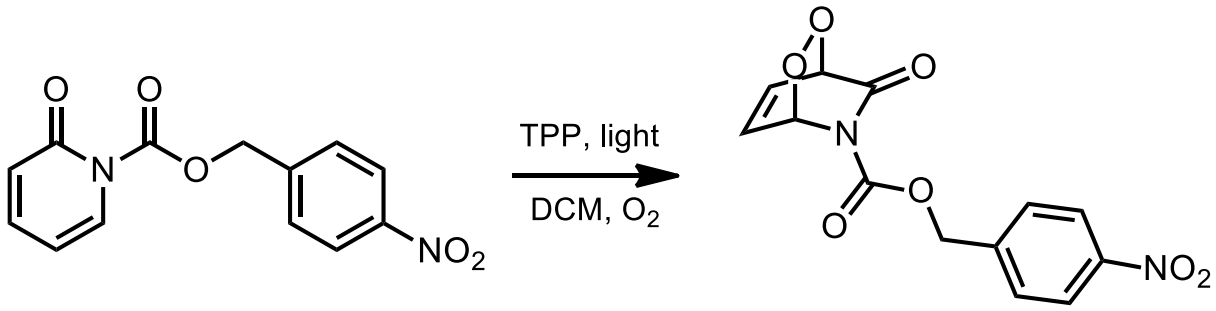
2.1. Synthetic Procedures

Synthesis of Compound 3



$Mg(Ot\text{-}Bu)_2$ (896 mg, 5.26 mmol, 1.05 eq), KOt-Bu (309.7 mg, 2.76 mmol, 1.05 eq) and 2-pyridone (250 mg, 2.63 mmol, 1 eq) were added to schelenk tube. After purged with argon, dry THF (3 mL) was added and stirred for 10 min. To the resulting mixture was charged 4-nitrobenzyl chloroformate (850 mg, 3.94 mmol, 1.5 eq). The resulting mixture was stirred at room temperature for 2.5 h and then heated at 35 °C for 48 h. The reaction mixture was cooled to room temperature and quenched with HCl (3M, 6 mL). The mixture was extracted with EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by column chromatography and then preparative thin layer chromatography using DCM:MeOH (98:2, v/v). Compound **3** was obtained as yellowish solid (64 mg, 9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.22 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.8 Hz, 2H), 7.42-7.38 (m, 1H) 7.34-7.30 (m, 1H), 6.66-6.60 (m, 1H), 6.26-6.20 (m, 1H), 5.25 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 139.9, 137.1, 128.6, 124.1, 121.7, 106.7, 51.8. MS (TOF-ESI) m/z calcd for $C_{13}H_{10}N_2O_5$: 275.06625 $[M+H]^+$, found 275.06354 $[M+H]^+$, Δ= 9.84 ppm

Synthesis of Endoperoxide 1



Compound **3** (33.4 mg, 0.122 mmol) was dissolved in DCM. Tetraphenylporphyrin (TPP) (~5.0 mg) was added to the reaction mixture at -78 °C which was irradiated while oxygen gas was passing through it. The progress of the reaction was monitored by TLC. When TLC showed no starting material, the mixture was concentrated in vacuo at 25 °C and the residue was purified by silica gel flash column chromatography with DCM:MeOH (99:1, v/v) as the eluent. Endoperoxide **1** was obtained as pale yellowish solid (30 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, *J* = 8.8 Hz, 2H), 7.43 (d, *J* = 8.8 Hz, 2H), 6.85-6.81 (m, 2H), 5.61-5.63 (m, 1H), 5.15-5.13 (m, 1H), 4.83 (d, *J* = 16.0 Hz, 1H), 4.68 (d, *J* = 16.0 Hz, 1H). MS (TOF-ESI) m/z calcd for $C_{13}H_{10}N_2O_7$: 305.04152 $[M-H]^-$, found 305.04066 $[M-H]^-$, Δ= 2.83 ppm

2.2. Characterization Data

^{1}H NMR and ^{13}C NMR Spectra

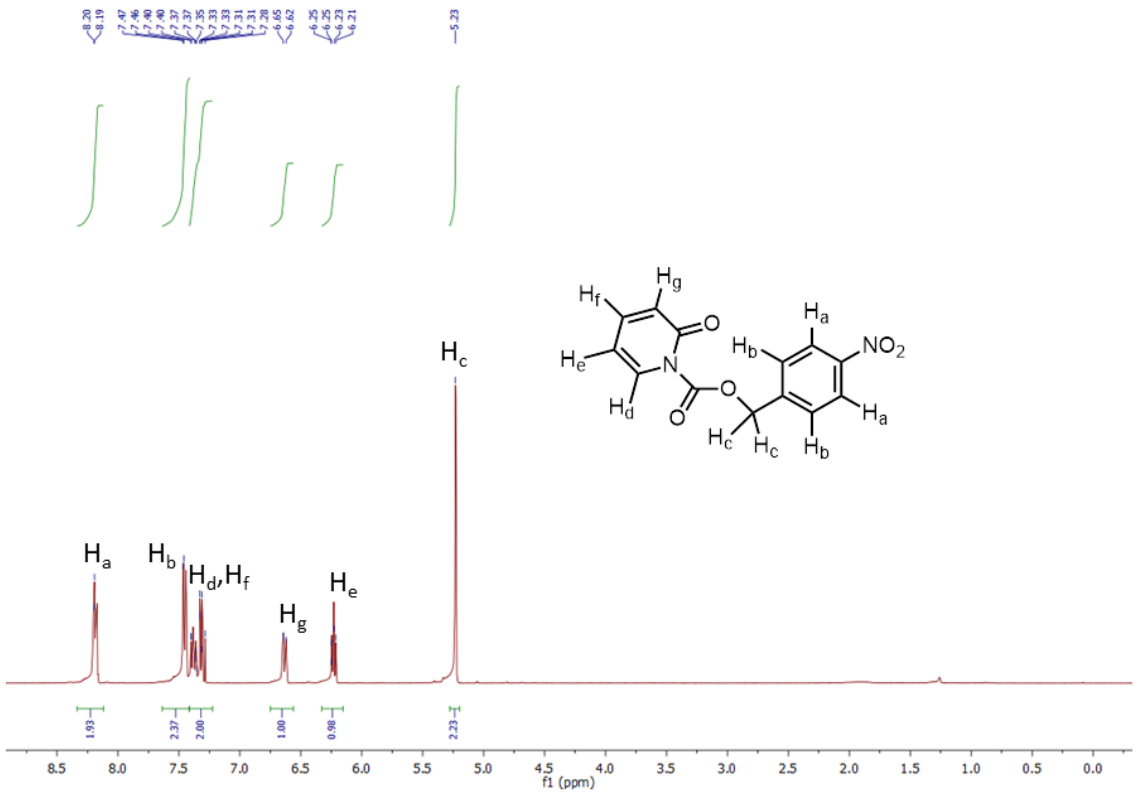


Figure S1. ^{1}H NMR Spectrum of Compound **3**

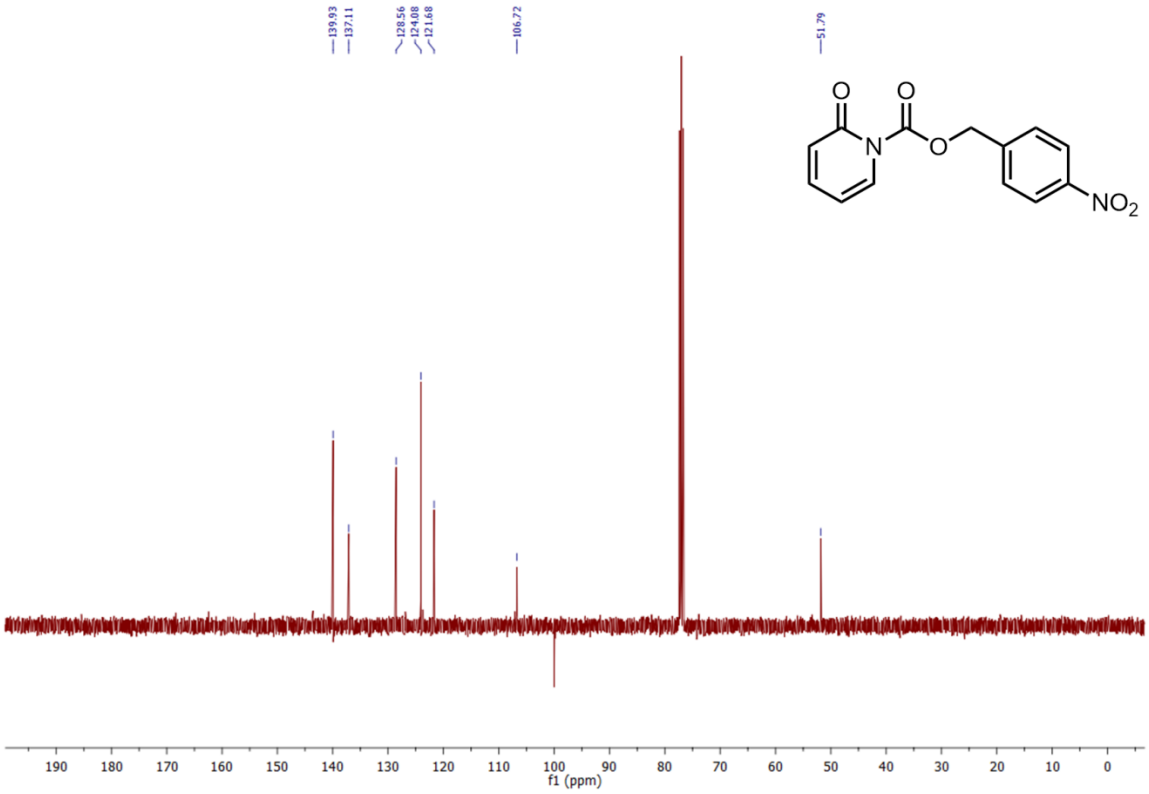


Figure S2. ^{13}C NMR Spectrum of Compound **3**

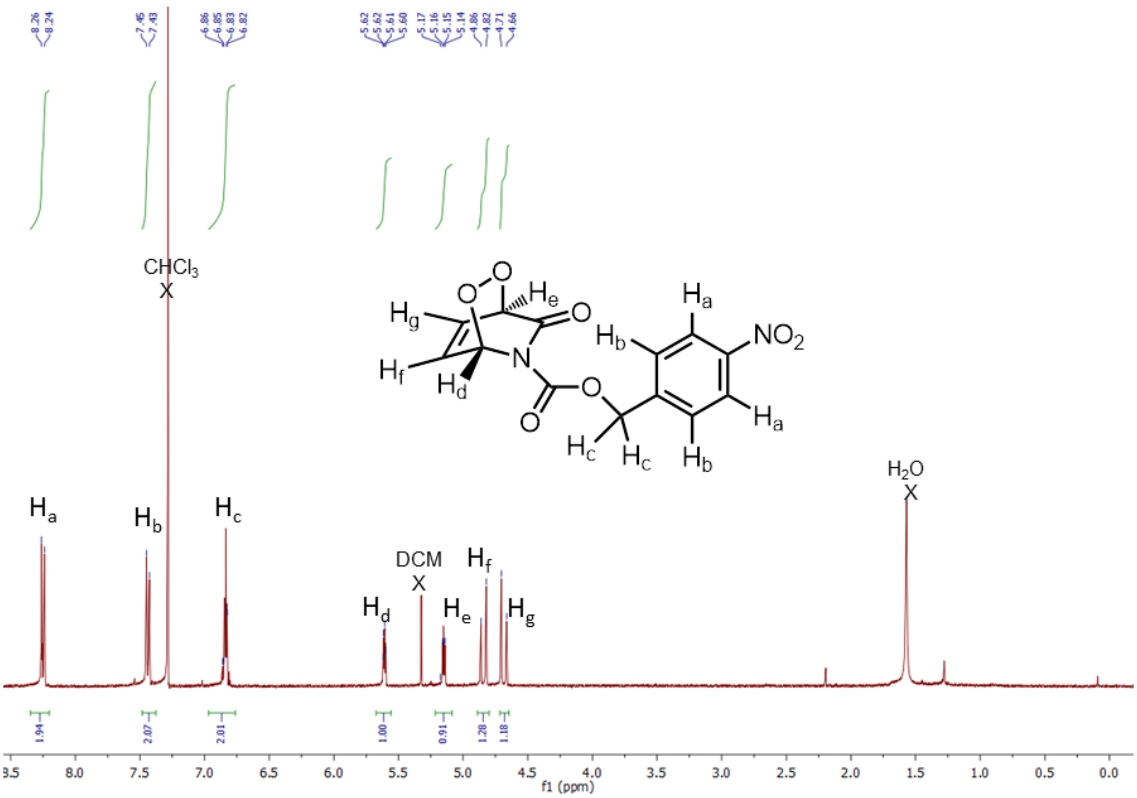


Figure S3. ^{1}H NMR Spectrum of Endoperoxide **1**

HRMS Spectra

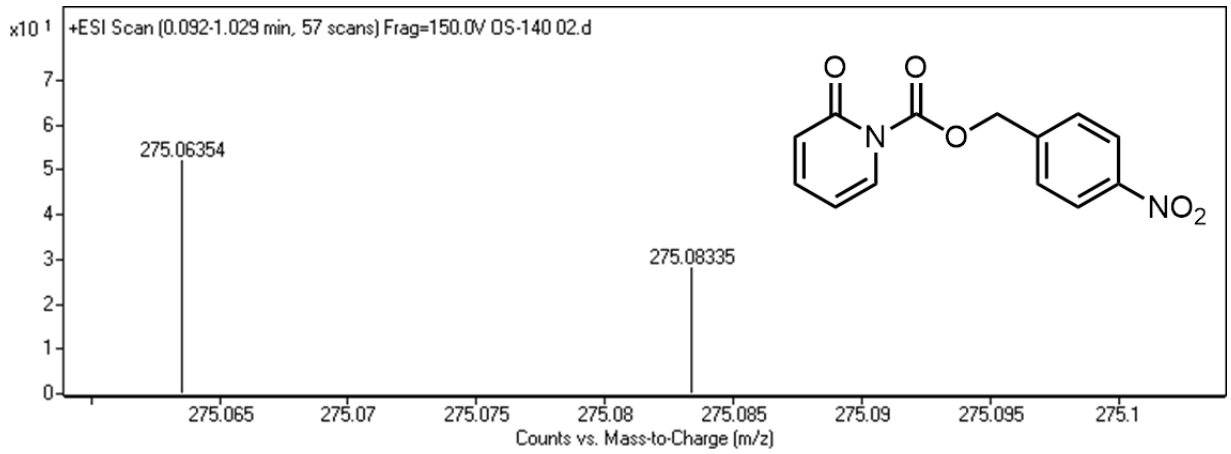


Figure S4. HRMS Spectrum of Compound **3**

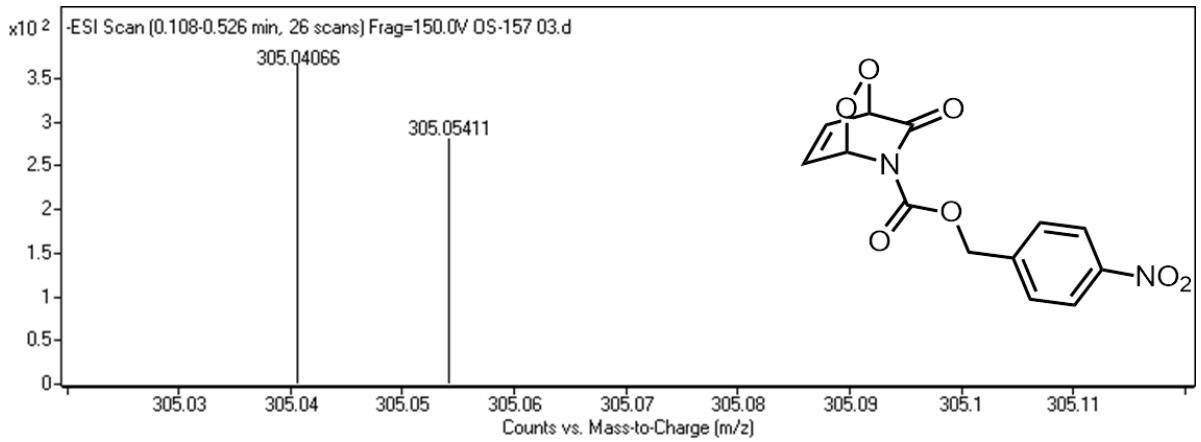


Figure S5. HRMS Spectrum of Endoperoxide **1**

3. Kinetic Experiments of the Endoperoxides

With the intention of getting more qualitative results, the NMR investigations of two systems were performed as a function of time, the solvent being $CDCl_3$. It was observed that the endoperoxide **1** had a half-life of **7.1** hours at 37 °C, while the endoperoxide **2** had a half-life of **1.3** hours according to appearance/disappearance of their normalized integral values of the selected peaks. The rate constant and half-life calculations were done in accordance to the first-order reaction rate equations. The equations are given below:

$$\ln[A] = -kt + \ln[A]_0 \quad , \quad t_{1/2} = 0.693/k \qquad \text{Equation (1)}$$

In the following NMR spectra, it is possible to observe the evolution of peaks (7.42-7.38, 7.31, 6.67, 6.25 ppm) which belong to compound **3** due to endoperoxide **1** cycloreversion. While the peaks of parent compound increase, the peaks of endoperoxide **1** (6.85-6.81, 5.62, 5.15-5.13 ppm) decrease.

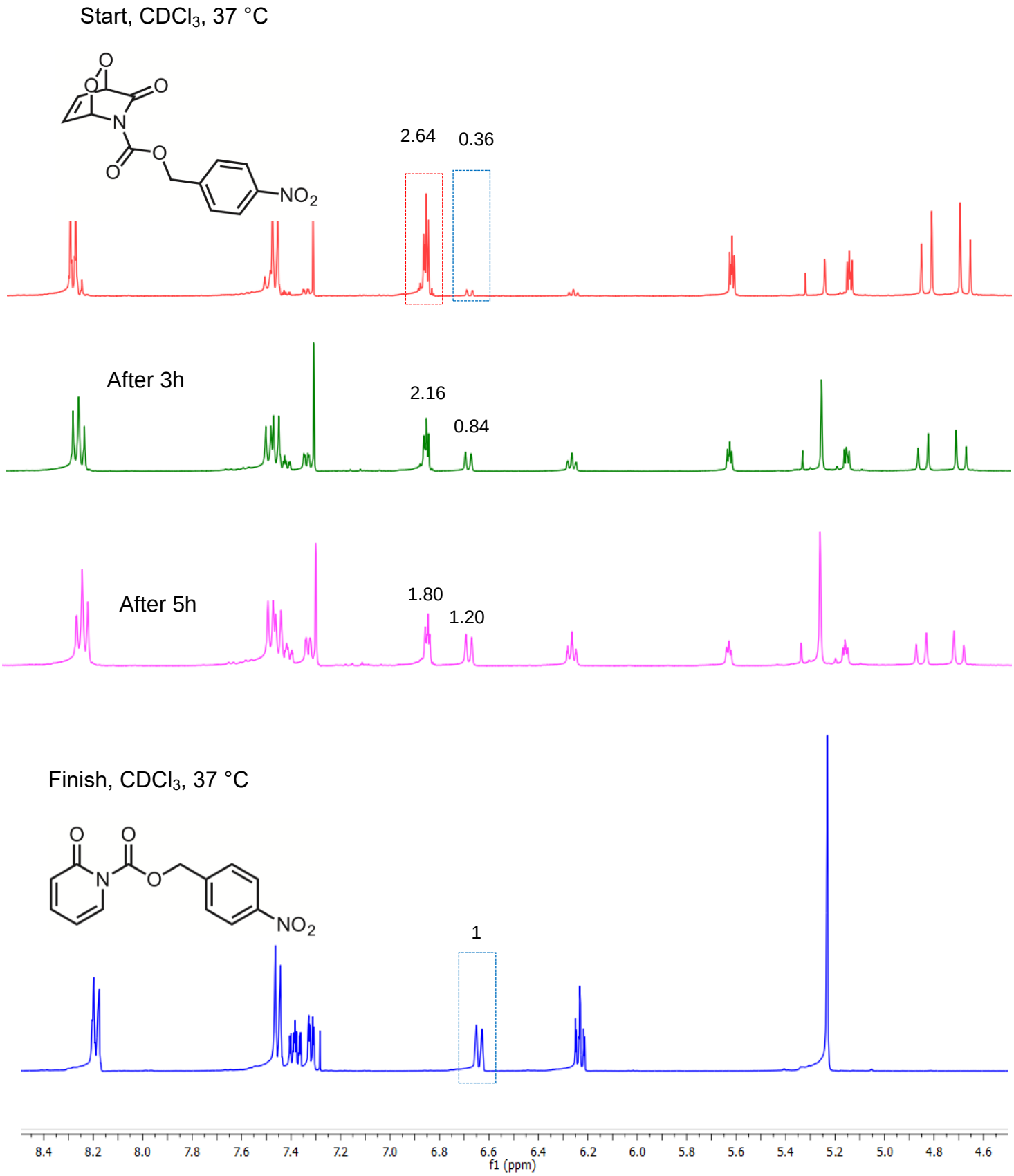


Figure S6. Evolution of the NMR spectra of cycloreversion reaction of endoperoxide **1** over time at 37 °C in $CDCl_3$ as the solvent, in order to

determine the half-life of endoperoxide **1**. (Spectra at 0 hours (endoperoxide **1**), Spectra after 3 hours, Spectra after 5 hours, Spectra of compound **3**)

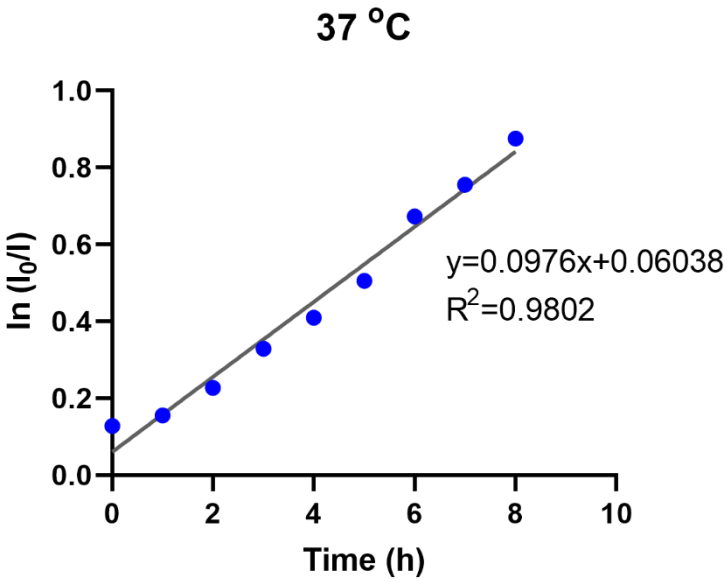


Figure S7. Cycloreversion of endoperoxide **1** over time at 37 °C. The values were obtained from ^{1}H NMR in $CDCl_3$. The half-life was calculated as 7.1 h according to the equation (1).

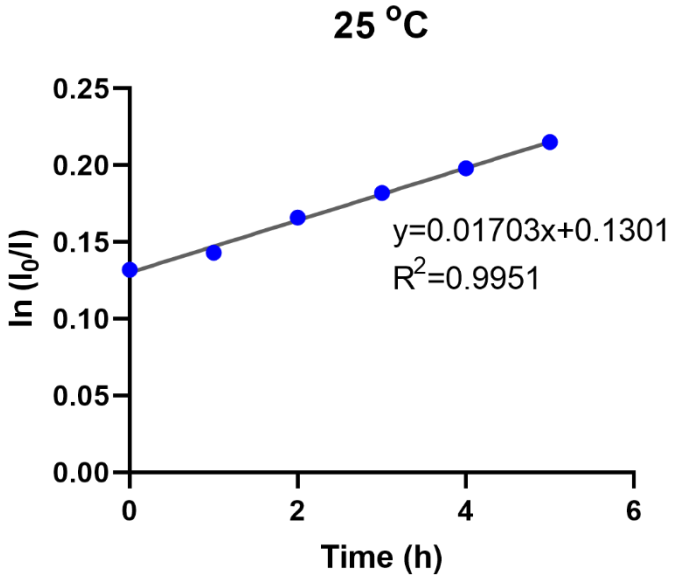


Figure S8. Cycloreversion of endoperoxide **1** over time at 25 °C. The values were obtained from ^{1}H NMR in $CDCl_3$. The half-life was calculated as 40.7 h according to the equation (1).

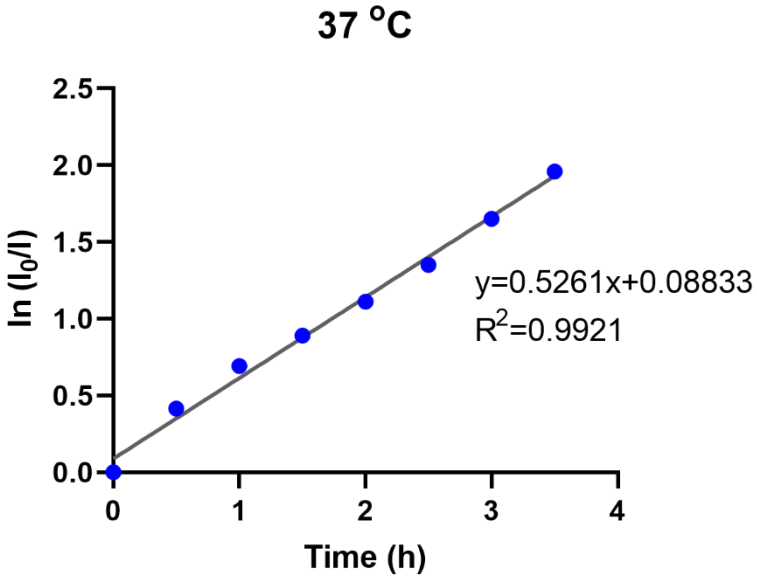


Figure S9. Cycloreversion of endoperoxide **2** over time at 37 °C. The values were obtained from ^{1}H NMR in $CDCl_3$. The half-life was calculated as 1.3 h according to the equation (1).

4. Cell Studies

4.1. Cell Culture

The sources for the cell lines are as follows:

MCF7 (obtained from ATCC)

K562 (obtained from ATCC)

HeLa (obtained from ATCC)

MRC-5 (obtained from ATCC)

MCF-12A (obtained from ATCC)

NIH/3T3 (obtained from ATCC)

HEK 293T (obtained from ATCC)

Nthy-ori 3-1 (obtained from ECACC)

In order to investigate the cellular effects of the endoperoxide **1**, cell culture assays were performed with a human breast adenocarcinoma cell line (MCF7), a human cancer suspension cell line-chronic myelogenous leukaemia (K562) and a human cervix cancer cell line (HeLa) as well as a human normal fibroblast cell line (MRC-5), a human non-tumorigenic breast cell line (MCF-12A), a mouse fibroblast cell line (NIH/3T3), a human embryonic kidney cell line (HEK 293T) and a human normal thyroid follicular cell line (Nthy-ori 3-1). MCF7, HeLa, MRC-5 and HEK 293T cells were incubated in 25 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS at the environmental conditions of 37 °C, 5 % CO_2 and 60 % humidity. K562 cells were incubated in 25 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % FBS at the environmental conditions of 37 °C, 5 % CO_2 and 60 % humidity. NIH/3T3 cells were incubated in 25 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 10 % FCS at the environmental conditions of 37 °C, 5 % CO_2 and 60 % humidity. MCF-12A cells were incubated in 25 cm^2 culture flasks with Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone and 5 % horse serum at the environmental conditions of 37 °C, 5 % CO_2 and 60 % humidity. Nthy-ori 3-1 cells were incubated in 25 cm^2 culture flasks with Roswell Park Memorial Institute Medium (RPMI 1640) supplemented with 10 % FBS at the environmental conditions of 37 °C, 5 % CO_2 and 60 % humidity. Cells were exposed to varying concentrations of the endoperoxide **1** and incubated under conditions of either normoxia (21% O_2, 5% CO_2, 74% N_2) or hypoxia (0.5% O_2, 5% CO_2, 94.5% N_2). Untreated samples were used as negative controls for both of the drug treatments under hypoxia or normoxia.

4.2. Hypoxia Treatment

In order to simulate hypoxic conditions, cells were placed in a humidified modular incubator chamber (Billups-Rothenberg, Inc., USA)[1] containing 0.5% O_2, 5% CO_2 and 94.5% N_2 (v/v), by flushing the gas mixture for at least 6 min at a flow rate of 30 l min^{-1}. The modular incubator chamber was then sealed and placed in a conventional cell incubator at 37 °C. The chamber was re-flushed after 1 hour of incubating to remove any residual trapped gasses. Control cells were incubated in identical conditions for the same duration under normoxic conditions, 21% O_2, 5% CO_2 and 74% N_2 (v/v). When the cells reached to a confluency of >70% in the culture conditions, they were seeded in 96-well plates for the MTT (3-(4, 5-dimethylthiazolyl-2)−2,5-diphenyltetrazolium bromide) analyses and in E-Plates for the real-time and label-free analyses with the iCELLigence system. Cells were cultured for 24 h at

normoxic conditions in order to achieve proper adhesion of the cells to the plates. Subsequently, hypoxic group of the cells were incubated 24 h further under conditions of hypoxia (pre-hypoxia [without drug treatment]); whereas, normoxic group of the cells were kept under normoxic conditions for the same period of time.

4.3. MTT Assay

The main *in vitro* functional goal of the current study is to study the difference of cytotoxicity of endoperoxide **1** under hypoxic and normoxic conditions. Both hypoxia group and the normoxia group (at the end of the pre-hypoxia/pre-normoxia period) of the cells were treated with varying concentrations of endoperoxide **1**. The compound was diluted in cell culture medium and assay concentrations were freshly prepared. The hypoxia group of the cells were incubated further under conditions of hypoxia (with or without 100 µM L-Buthionine-sulfoximine [BSO] [Sigma-Aldrich, MO, USA]) with endoperoxide **1**; while normoxia group of the cells were incubated further under conditions of normoxia with endoperoxide **1**.

Untreated samples were used as negative controls for both of the drug treatments under hypoxia or normoxia. Cell viability / death was evaluated by MTT assay. Briefly, 50 µL cell suspensions in culture medium containing MCF7 or K562 cells were plated in 96-well flat-bottom culture plates (Corning, MA, USA) and incubated for 24 hours to recover from handling. Varying concentrations of the chemical compound in cell culture medium was added into each well (at the end of the pre-hypoxia/pre-normoxia period). The experimental group of the cells were incubated under hypoxic conditions, as explained in the “4.2. Hypoxia Treatment” section. The normoxia group of the cells were incubated in normoxic conditions, for the exact

duration under identical environmental conditions except O_2 tension. According to the assay protocol, 25 µL of the MTT reagent (Sigma-Aldrich, MO, USA) was added to each well in order to assess cell viability (final concentration: 1 mg/mL) at the end of the 24 h drug treatment period. Following a 4 h incubation of the cells with the MTT reagent, the generated formazan precipitates were solubilized by the addition of the lysing buffer (80 µL, pH: 4.7), which is composed of 23 % SDS (Sodium dodecyl sulfate) dissolved in a solution of 45 % DMF (N,N-Dimethylformamide). After an overnight incubation at 37 °C, the absorbance values (of each well) were measured at 570 nm in a microtiter plate reader (Spectramax Plus, Molecular Devices, CA, USA) at 25 °C. Cells incubated in culture medium only (without the compound) served as the control for cell viability for the plates incubated in either hypoxia or normoxia; whereas DMSO (50 %, v/v) was used to observe maximum cell death (positive control). Cell viabilities of normal cell lines were also evaluated by MTT assays (Figure S23).

The results of each of the groups (hypoxia [with or without BSO] and normoxia) were normalized to untreated negative control samples in either hypoxic (with or without BSO) or normoxic conditions. Therefore, the sole effect of hypoxic conditions (without drug treatment) on the cells could be eliminated and the observed effects in MTT results were made sure to be due to the effects of endoperoxide **1**, solely.

Glutathione (GSH) serves as a critical antioxidant in cells and it is capable of preventing damage by reactive oxygen species and the resulting free radical induced apoptosis.[2] Elevated GSH levels were reported in various types of tumors.[3] BSO, which is an inhibitor of GSH synthesis, may be used to increase the sensitivity of

cancer cells to oxidative drugs, such as endoperoxides. MCF7 cancer cells were shown to express high levels of GSH and their expression of GSH can be decreased via BSO treatment.[4] Therefore, MCF7 cells were also incubated with varying concentrations of endoperoxide **1** in the presence of BSO.

Lower doses of endoperoxide **1** seem to have resulted in a decrease of the cell viability under hypoxic conditions (Figure S10), in contrast to normoxic conditions. The IC_{50} values of the compound in the hypoxic and normoxic conditions were estimated by fitting a model with non-linear regression. On the contrary, control compound **3** does not cause any cytotoxicity on cancer cells under conditions of hypoxia or normoxia (Figure S11).

4.4. Real-time and Label-free Analyses of Cells with the iCELLigence System

In order to reveal the temporal profile of the endoperoxide **1** responses in real time, allowing for the observation of both acute responses and longer-term responses within the same assay; we performed functional analyses with the RTCA iCELLigence System (ACEA, San Diego, CA, USA).[5] This approach provides a method for spatial and temporal dynamic view of the cell populations with the effect of endoperoxide **1** under hypoxic or normoxic conditions. As a result, both the short- and long-term effects of endoperoxide **1** under hypoxia and normoxia could be visualized, at an unprecedented level of detail. The system utilizes gold biosensor microelectrodes to measure electrical impedance. The background impedances of the microelectrodes were first measured with cell culture medium. Then, MCF7 cancer cells were added to each well of the E-Plate and cultured for 24 h at normoxic conditions in order to achieve proper adhesion of the cells to the E-Plates.

Subsequently, hypoxic group of the cells were incubated 24 h further under conditions of hypoxia (pre-hypoxia [without drug treatment]); whereas, normoxic group of the cells were kept under normoxic conditions for the same period of time (as explained in the “4.2. Hypoxia Treatment” section). At the end of this period, both groups of the cells were treated with varying concentrations of endoperoxide **1**. The hypoxia group of the cells were incubated further under conditions of hypoxia with endoperoxide **1**; while normoxia group of the cells were incubated further under conditions of normoxia with endoperoxide **1**. The cell indices were measured by electrical impedance over an additional period of 24 hours. Untreated samples were used as negative controls for both of the drug treatments under hypoxia or normoxia. The results of each of the groups (hypoxia and normoxia) were normalized to relevant untreated negative control samples in either hypoxic or normoxic conditions, and the results were shown as a “% of Control”. Therefore, the sole effect of hypoxic conditions (without drug treatment) on the cells could be eliminated and the observed effects in iCELLigence results were made sure to be due to the effects of endoperoxide **1**, exclusively.

Lower doses of endoperoxide **1** seem to have resulted in a significant decrease of the cell viability under hypoxic conditions at a much earlier time (Figure 4), in contrast to normoxic conditions. (Figure S13 demonstrates iCELLigence results for HeLa cells.) The KT_{50} (the amount of time it takes to kill 50 % of the cancer cells) values of the 50 µM endoperoxide **1** in the hypoxic and normoxic conditions were approximately 59.5 h and 212 h, respectively. It is important to note the acute effect of the compound seen under hypoxia at 50 µM (4-12 h) in contrast to normoxia. In summary, our results show the value of such a real-time approach in measuring the

cytotoxic response across the temporal scale, clearly demonstrating the value of endoperoxide **1** at a temporal dimension.

4.5. Flow Cytometry

In order to confirm our cytotoxicity results, Annexin V detection protocol for apoptosis using flow cytometry was performed. Annexin V flow cytometric analysis on adherent cell types (*e.g.* MCF7, HeLa) is not routinely tested; since specific membrane damage may occur during cell detachment or harvesting. Therefore, Annexin V flow cytometric analyses were performed with a human cancer suspension cell line-chronic myelogenous leukaemia (K562). K562 cells treated with endoperoxide **1** under either hypoxic or normoxic conditions were stained with Annexin V (Fluorescein isothiocyanate-FITC), as described in the technical data sheet (BD Pharmingen, USA), and were analysed by FACS Aria II (equipped with 488 nm and 635 nm lasers) using FACS Diva software. The percentage of fluorochrome-labelled Annexin V (FITC) stained cells analysed with flow cytometry was higher in the cells treated with endoperoxide **1** under hypoxic conditions compared with the ones incubated under normoxic conditions (25.3 % vs. 16.0 % at 25 μM and 18.3% vs. 6.75% at 12.5 μM; Figures S14-19). Our Annexin V analyses with flow cytometry confirmed both the MTT and iCELLigence results, concerning the difference of effects under hypoxia vs. normoxia. In addition, analyses of cell viability by Trypan blue stain further confirmed these results (Figure S21).

4.6. Microscopy (Detection of Singlet Oxygen Generation)

In order to assess the singlet oxygen generation of endoperoxide **1** under normoxic or hypoxic conditions, we performed fluorescence imaging by using a cell permeable

reactive oxygen species (ROS) sensor 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA). HeLa cells were cultured in 35-mm glass bottomed dishes at a density of 20,000 cells per dish in 1.8 mL of full growth media (DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin) at 37 °C with 5 % CO_2 for 24 h at normoxic conditions in order to achieve proper adhesion of the cells to the glass bottomed dishes. Subsequently, hypoxic group of the cells were incubated 24 h further under conditions of hypoxia (pre-hypoxia [without drug treatment]); whereas, normoxic group of the cells were kept under normoxic conditions for the same period of time (as explained in the "4.2. Hypoxia Treatment" section). At the end of this period, the cells were washed with phosphate-buffered saline (PBS) and then treated with 1.8 mL of 10 µM $DCFH_2$ solution (0.2 % DMSO) in PBS and incubated for 45 minutes at 37 °C. The solution was removed and the cells were washed one time with PBS. Both normoxic and hypoxic groups of the cells were then treated with 50 µM endoperoxide **1**. The hypoxia group of the cells were incubated further under conditions of hypoxia for 3 h with endoperoxide **1**; while normoxia group of the cells were incubated further under conditions of normoxia for 3 h with endoperoxide **1**. A group of the cells kept under normoxic conditions and a group of the cells kept under hypoxic conditions were treated with full growth media containing 10 mM NaN_3 as well as 50 µM endoperoxide **1** in order to test singlet oxygen generation; since NaN_3 is known to reduce singlet oxygen generation. In addition, groups of the cells that were incubated only with 10 µM $DCFH_2$ solution (without endoperoxide **1**) under hypoxic and normoxic conditions were utilized as control groups. After 3 h, the media were removed and replaced with 1.8 mL of PBS for imaging via microscopy. Both PBS and growth media were warmed to 37 °C prior to their addition to cells. Microscopy images were acquired using a Leica THUNDER Imager (3D Live Cell &

3D Cell Culture & 3D Assay). Cells were observed under ×10 objective and a Green Fluorescent Protein filter set was used to visualize the green fluorescence emitted by DCF (which is produced upon reaction with ROS).

5. Figures

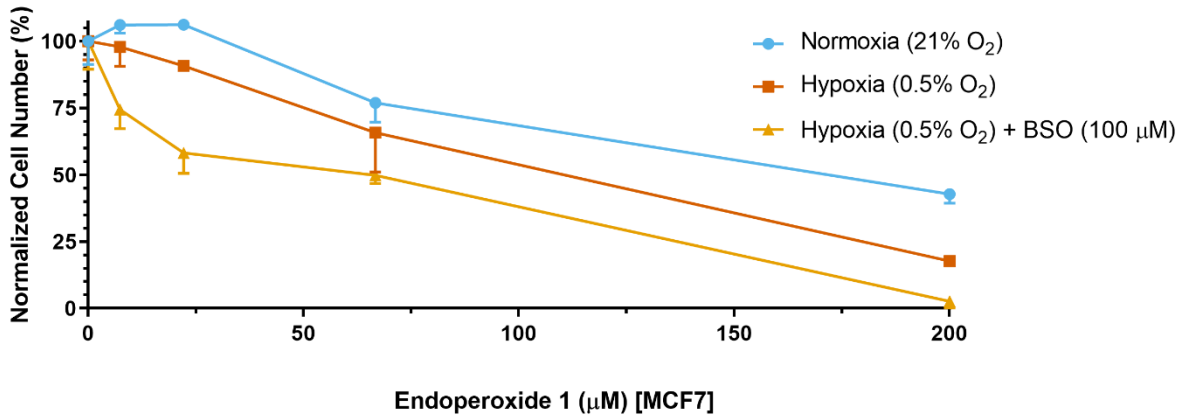


Figure S10. Endoperoxide 1 achieves potent cytotoxicity on cancer cells under conditions of hypoxia. Cell viabilities of MCF7 breast cancer cells were evaluated with MTT assay after 24 hours of treatment with varying concentrations of endoperoxide **1** under either normoxic (IC_{50}: 162 µM) or hypoxic (without BSO IC_{50}: 91 µM, with BSO IC_{50}: 34 µM) conditions; and normalized cell numbers are shown (points and error bars designate means and SD, respectively). Red line corresponds to normalized cell numbers of MCF7 cells under hypoxic conditions. Blue line corresponds to cells kept under identical conditions of incubation with endoperoxide **1**, but under normoxic conditions. Orange line corresponds to normalized cell numbers of MCF7 cells under hypoxic conditions with 100 µM BSO treatment. Negative control corresponds to cells incubated in complete growth medium, without endoperoxide **1** treatment.

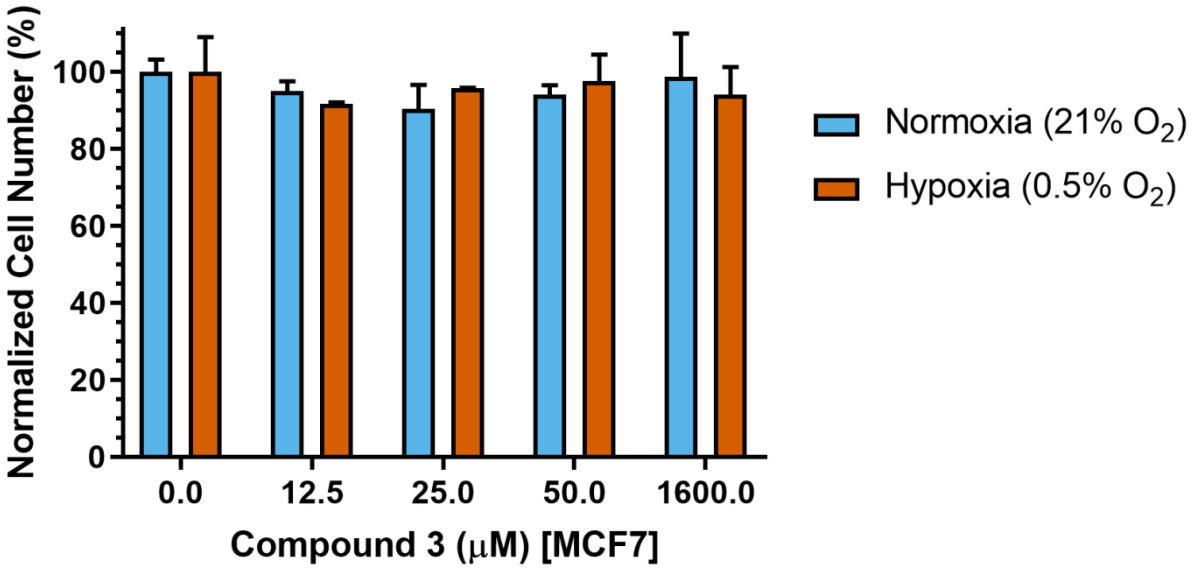


Figure S11. The control compound 3 had no significant toxicity either under hypoxic or normoxic conditions, even at very high concentrations of 1.6 mM. Cell viabilities of MCF7 breast cancer cells in the presence of compound **3** were determined with MTT assays after 24 hours of treatment with varying concentrations of compound **3** under either normoxic or hypoxic conditions; in order to eliminate any complications that may arise from a possible cytotoxicity of the compound **3** and any other reduction by-product. Normalized cell numbers are shown (coloured bars and error bars designate means and SD, respectively). Red bars correspond to normalized cell numbers of MCF7 cells under hypoxic conditions. Blue bars correspond to cells kept under identical conditions of incubation with the compound **3**, but under normoxic conditions. Negative control corresponds to cells incubated in complete growth medium, without compound **3** treatment.

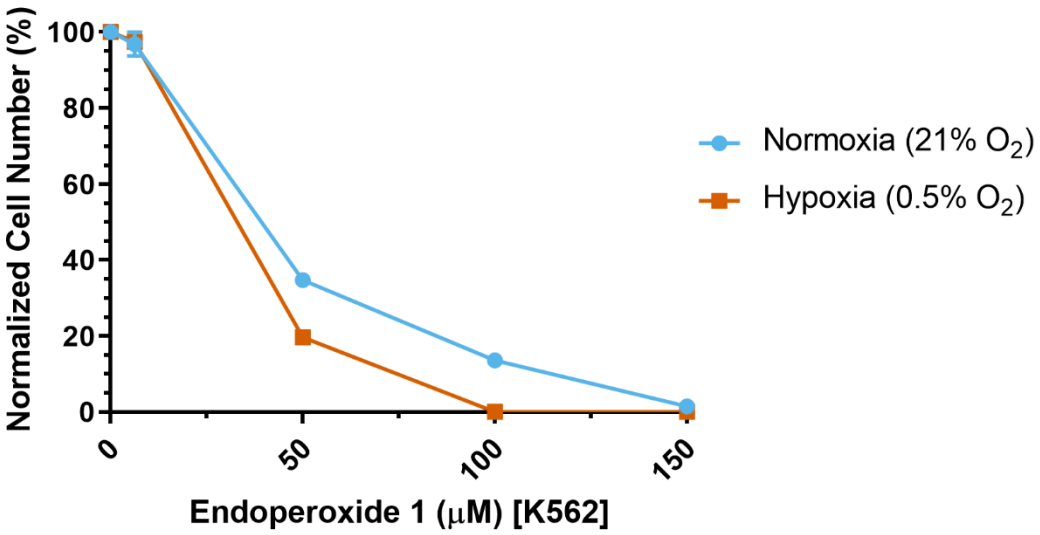


Figure S12. Endoperoxide 1 achieves potent cytotoxicity on cancer cells under conditions of hypoxia. Cell viabilities of K562 human chronic myelogenous leukaemia cells were evaluated with MTT assay after 24 hours of treatment with varying concentrations of endoperoxide **1** under either normoxic (IC_{50}: 40 µM) or hypoxic (IC_{50}: 28 µM) conditions; and normalized cell numbers are shown. Red line corresponds to normalized cell number of K562 cells under hypoxic conditions. Blue line corresponds to cells kept under identical conditions of incubation with the endoperoxide **1**, but under normoxic conditions. Negative control corresponds to cells incubated in complete growth medium, without endoperoxide **1** treatment.

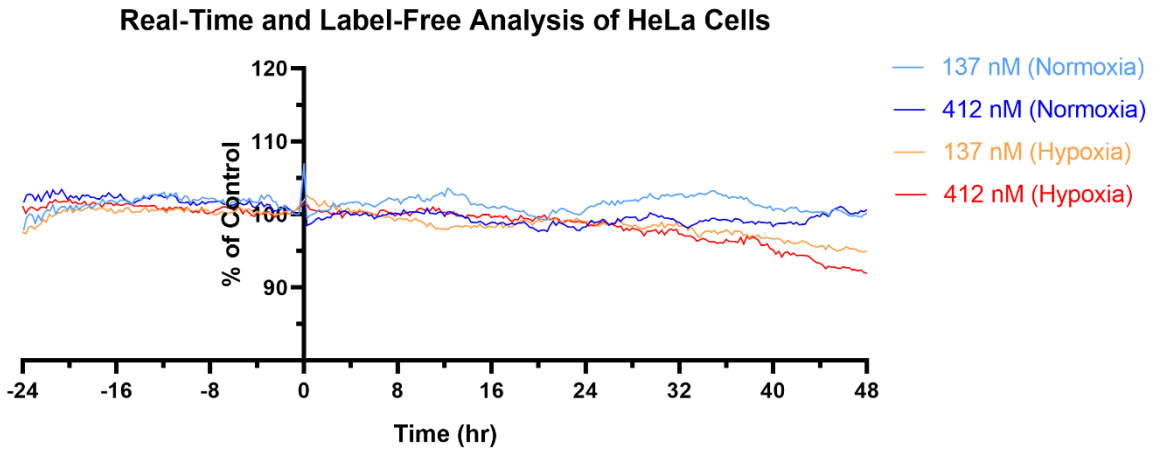


Figure S13. The temporal profile of the effects of endoperoxide 1 on HeLa cancer cells. Coloured lines correspond to normalized cell numbers of HeLa cells treated with endoperoxide **1** under hypoxic or normoxic conditions (red: 412 nM in hypoxia, orange: 137 nM in hypoxia, blue: 412 nM in normoxia, turquoise: 137 nM in normoxia). Control corresponds to cells incubated in complete growth medium, without endoperoxide **1** treatment, under either normoxic or hypoxic conditions. The results of each of the groups (hypoxia and normoxia) were normalized to relevant untreated control samples in either hypoxic or normoxic conditions, and the results were shown as a "% of Control" (drug treatments were initiated at 0 h; thus, 0 h was used as the time for temporal normalization). The KT_{50} (the amount of time it takes to kill 50 % of the cancer cells) values of the 412 nM and 137 nM endoperoxide **1** in hypoxic conditions were estimated approximately as 108.5 h and 264.5 h, respectively. The KT_{50} values of 412 nM and 137 nM endoperoxide **1** in the normoxic conditions cannot be estimated since their projections are way out of the scale of the model fit.

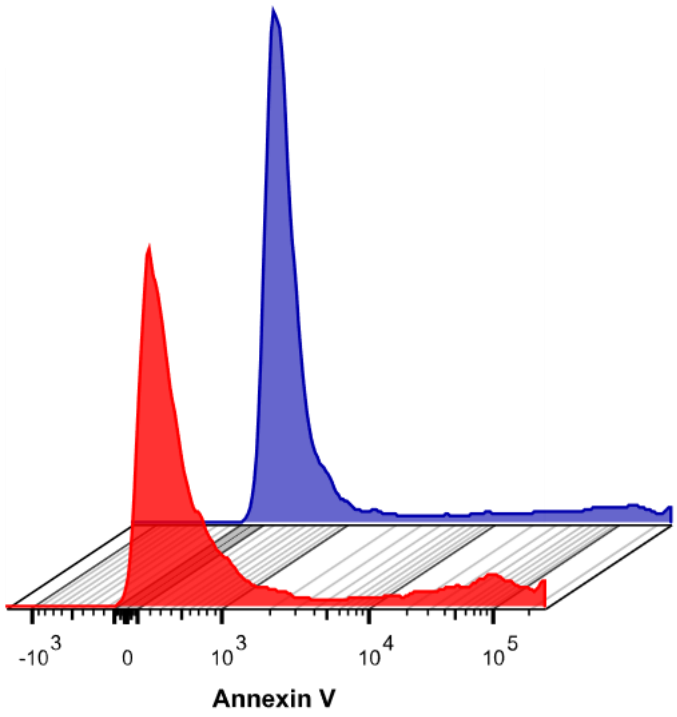


Figure S14. Endoperoxide 1 potently induces apoptosis in cancer cells under conditions of hypoxia. The percentage of FITC-Annexin V stained cells analysed with flow cytometry was higher in K562 cells treated with 25 µM endoperoxide **1** under hypoxic conditions compared with the cells incubated under normoxic conditions (25.3 % vs. 16.0% at 25 µM). Blue shaded area represents the cells incubated under normoxic conditions, red shaded area represents the cells incubated under hypoxic conditions in the overlay graph.

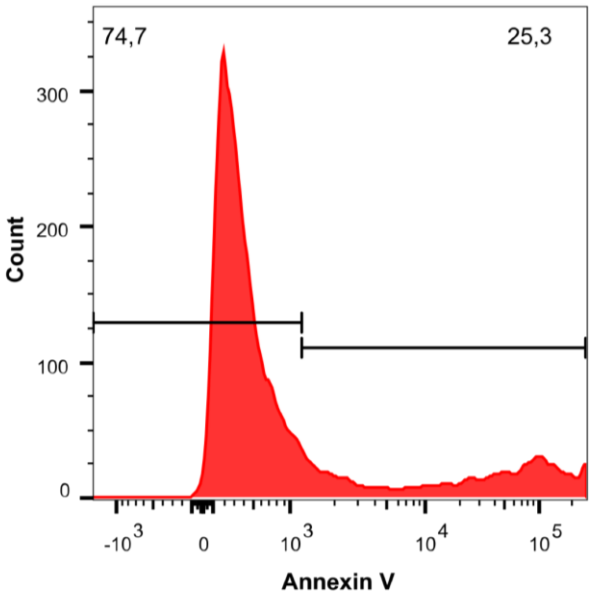


Figure S15. Endoperoxide 1 potently induces apoptosis in cancer cells under conditions of hypoxia. The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 25.3 % in the cells treated with 25 µM endoperoxide **1** under hypoxic conditions. Red shaded area represents the cells incubated under hypoxic conditions in the histogram.

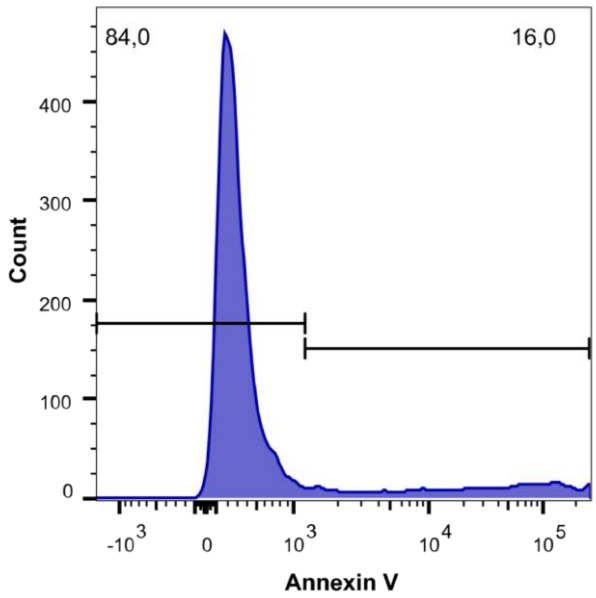


Figure S16. The effect of endoperoxide 1 on apoptosis in cancer cells under conditions of normoxia. The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 16.0 % in the cells treated with 25 µM endoperoxide **1** under normoxic conditions. Blue shaded area represents the cells incubated under normoxic conditions in the histogram.

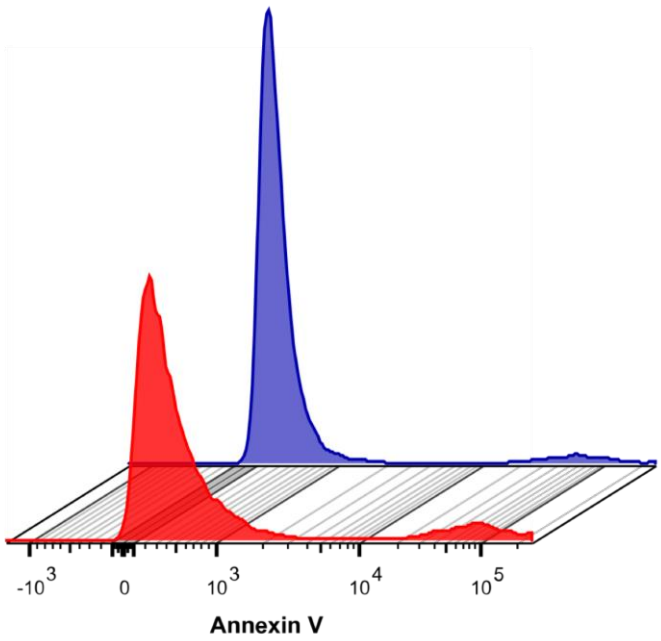


Figure S17. Endoperoxide 1 potently induces apoptosis in cancer cells under conditions of hypoxia. The percentage of FITC-Annexin V stained cells analysed with flow cytometry was higher in K562 cells treated with 12.5 µM endoperoxide **1** under hypoxic conditions compared with the cells incubated under normoxic conditions (18.3% vs. 6.75% at 12.5 µM). Blue shaded area represents the cells incubated under normoxic conditions, red shaded area represents the cells incubated under hypoxic conditions in the overlay graph.

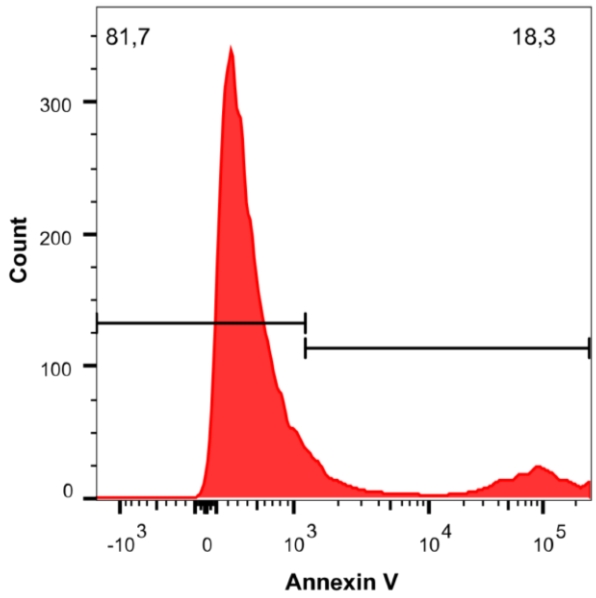


Figure S18. Endoperoxide 1 potently induces apoptosis in cancer cells under conditions of hypoxia. The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 18.3 % in the cells treated with 12.5 µM endoperoxide **1** under hypoxic conditions. Red shaded area represents the cells incubated under hypoxic conditions in the histogram.

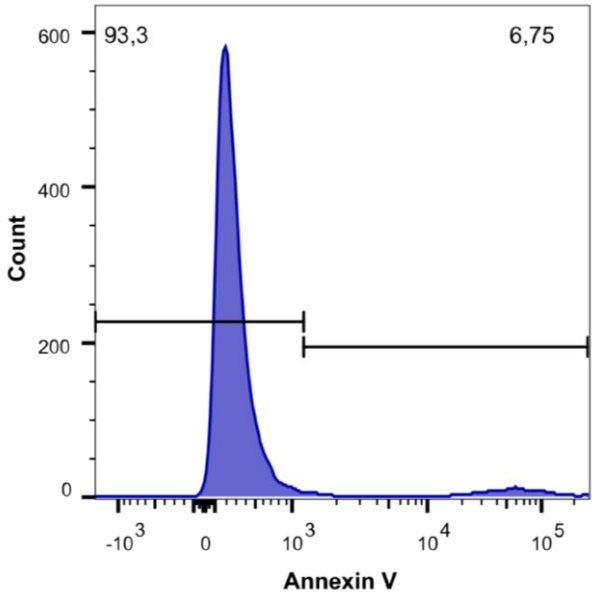


Figure S19. The effect of endoperoxide 1 on apoptosis in cancer cells under conditions of normoxia. The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 6.75 % in the cells treated with 12.5 µM endoperoxide **1** under normoxic conditions. Blue shaded area represents the cells incubated under normoxic conditions in the histogram.

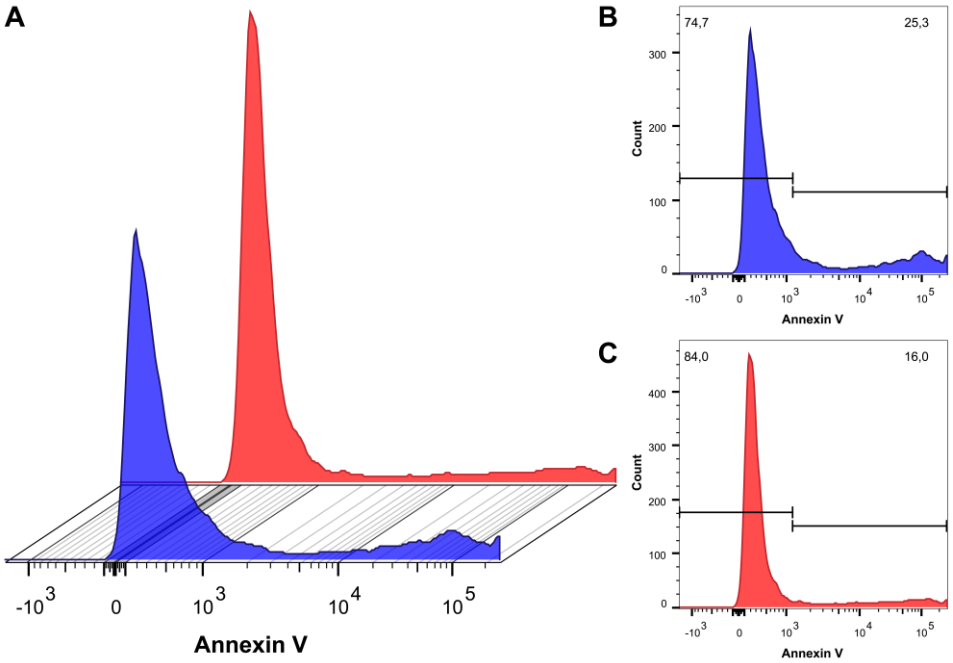


Figure S20. Endoperoxide 1 potently induces apoptosis in cancer cells under conditions of hypoxia. (A) The percentage of FITC-Annexin V stained cells analysed with flow cytometry was higher in K562 cells treated with 25 µM endoperoxide **1** under hypoxic conditions compared with the cells incubated under normoxic conditions. Blue shaded area represents the cells incubated under hypoxic conditions, red shaded area represents the cells incubated under normoxic conditions in the overlay graph. (B) The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 25.3 % in the cells treated with 25 µM endoperoxide **1** under hypoxic conditions. (C) The percentage of FITC-Annexin V stained K562 cells analysed with flow cytometry was 16.0 % in the cells treated with 25 µM endoperoxide 1 under normoxic conditions.

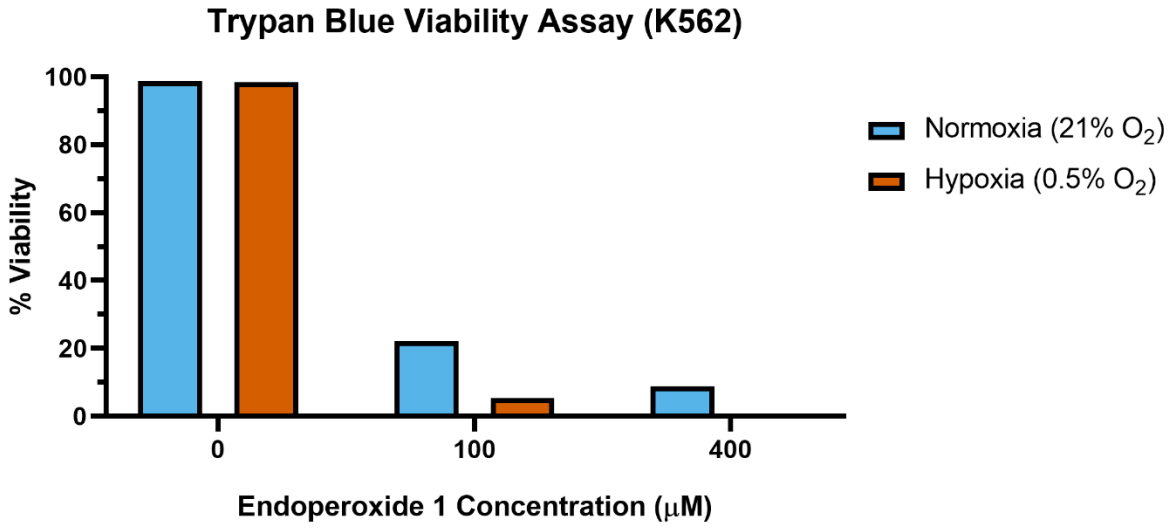


Figure S21. Analysis of K562 cell viability by Trypan blue stain demonstrated that endoperoxide 1 achieves potent cytotoxicity on cancer cells under conditions of hypoxia. Cell viabilities of K562 cells were evaluated with Trypan blue analysis after 24 hours of treatment with varying concentrations of endoperoxide **1** under either normoxic or hypoxic conditions. Red bars correspond to the percentages of viable K562 cells under hypoxic conditions. Blue bars correspond to the percentages of viable K562 cells kept under identical conditions of incubation with endoperoxide **1**, but under normoxic conditions. After a day of incubation under normoxic conditions, hypoxic group of the cells were incubated 24 h further under conditions of hypoxia (pre-hypoxia [without drug treatment]); whereas, normoxic group of the cells were kept under normoxic conditions for the same period of time. At the end of this period, both groups of the cells were treated with varying concentrations of endoperoxide **1**. The hypoxia group of the cells were incubated further under conditions of hypoxia with endoperoxide **1**; while normoxia group of the cells were incubated further under conditions of normoxia with endoperoxide **1**. Cells were then harvested in PBS and stained with the Trypan Blue stain. Unstained (live) and stained/blue (dead) cells were counted on an Improved Neubauer

haemocytometer. The percentage of live cells (number of live cells divided by the total number of cells) was plotted for each condition.

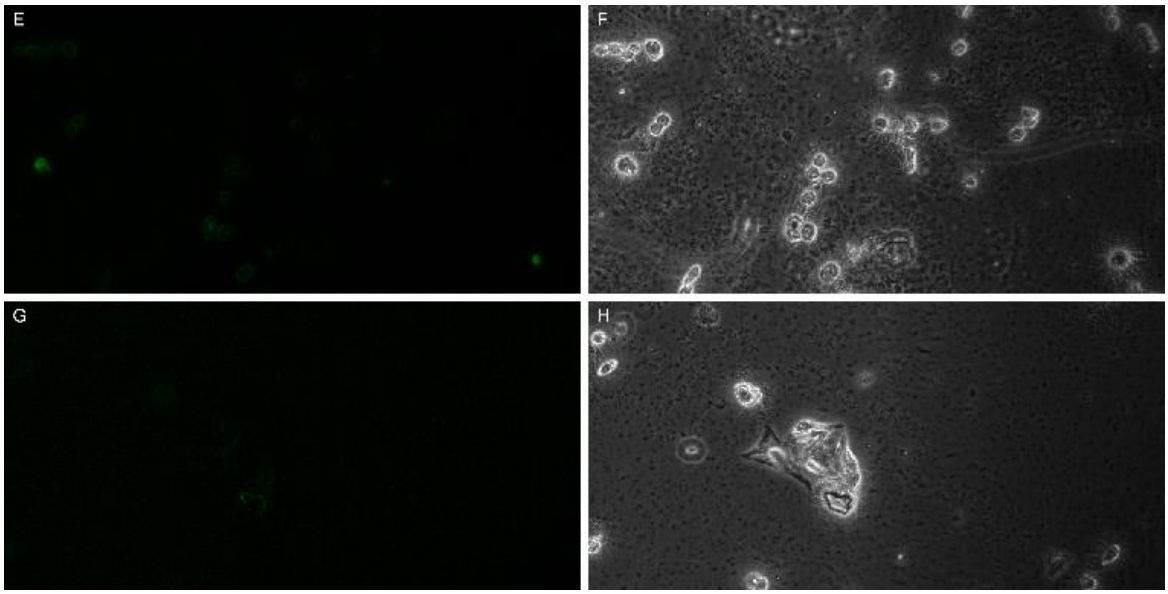


Figure S22. Singlet oxygen generation by endoperoxide 1 under normoxic and hypoxic conditions *in vitro* was analysed using cell permeable reactive oxygen species (ROS) sensor ($DCFH_2$-DA).

(**E**) Control group of the HeLa cells that were incubated only with 10 µM ROS sensor $DCFH_2$ (without endoperoxide **1**) under normoxic conditions. (**F**) Phase-contrast microscopy image of the cells in (**E**). (**G**) Control group of the HeLa cells that were incubated only with 10 µM ROS sensor $DCFH_2$ (without endoperoxide **1**) under hypoxic conditions. (**H**) Phase-contrast microscopy image of the cells in (**G**). Cells were observed under ×10 objective and a Green Fluorescent Protein filter set was used to visualize the green fluorescence emitted by DCF.

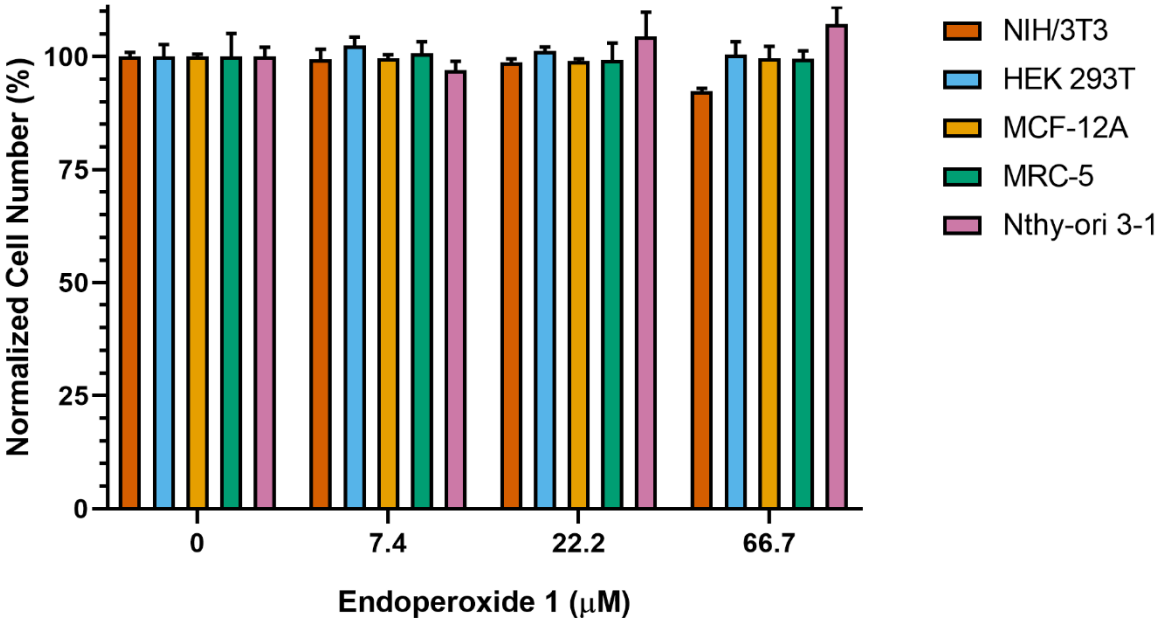


Figure S23 Endoperoxide **1** had no significant cytotoxicity on normal cells. Cell viabilities of NIH/3T3, HEK 293T, MCF-12A, MRC-5 and Nthy-ori 3-1 cells were evaluated with MTT assays after 24 hours of treatment with varying concentrations of endoperoxide **1**. Normalized cell numbers are shown (coloured bars and error bars designate means and SD, respectively).

6. References

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