Electronic Supplementary Information

Highly cytotoxic iron(II) complexes with pentadentate pyridyl ligands as a new class of anti-tumor agents

Ella Lai-Ming Wong, Guo-Su Fang, Chi-Ming Che* and Nianyong Zhu

Materials

All chemicals were analytical grade and purchased from Sigma-Aldrich Chemical Co. unless otherwise noted. Acetonitrile was pretreated with $KMnO_4$ and freshly distilled from CaH_2 before use. Analytical grade organic solvents and Milli-Q water were used throughout the experiments. Ligands 2,2′:6′,2″:6″,2‴-quaterpyridine (QP), 2,2′:6′,2″:6″,2‴;6‴,2⁗-quinquepyridine (qpy) and 2,6-bis[hyroxybis(2-pyridyl)methyl]pyridine (Py_5-OH) were synthesized and purified by the literature methods [E. C. Constable, S. M. Elder, M. J. Hannon, A. Martin, P. R. Raithby, D. A. Tocher, *J. Chem. Soc., Dalton Trans.*, 1996, 2423; E. C. Constable, M. A. M. Daniels, M. G. B. Drew, D. A. Tocher, J. V. Walker, P. D. Wood, *J. Chem. Soc., Dalton Trans.*, 1993, 1947; R. T. Jonas, T. D. P. Stack, *J. Am. Chem. Soc.*, 1997, **119**, 8566].

Supercoiled plasmid DNA pCR 2.1 was purchased from Invitrogen, and prepared and purified by maxi preparation according to standard procedures [Molecular Cloning A Laboratory Manual, 2nd Ed. Cold Spring Laboratory Press: Plainview, New York, 1989].

Human cervical epithelioid carcinoma (HeLa), human hepatocellular carcinoma

(HepG2), human hepatocellular carcinoma with p53 being knock out (Hep3B), human mammary gland adenocarcinoma (MCF-7) and normal human lung fibroblast (CCD-19Lu) cell lines were obtained commercially from American Type Culture Collection (ATCC). Human nasopharyngeal carcinoma (SUNE-1) cells were derived from poorly differentiated nasopharyngeal carcinoma (NPC) cells in Chinese patients [S. Y. Gu, W. P. Tang, Y. Zeng, E. W. Zhao, W. H. Deng, K. Li, *Chin, J. Cancer*, 1983, **2**, 270] and was generously provided by Dr. S. W. Tsao (Department of Anatomay, The University of Hong Kong, Hong Kong SAR, China). Taxol-resistant cell line, QGY-TR50, was derived from a human hepatocellular carcinoma QGY-7703 cell line [J. B. Wang, *Chung Hua Chung Liu Tsa Chih*, 1981, **3**, 241] and was generously provided by Prof. Y. Xie (Department of Biology, Hong Kong University of Science and Technology, Hong Kong SAR, China). Cell culture flasks, 6-well and 96-well microtitre plates were purchased from Nalge Nunc Int. 96-Well microtitre plates specially designed for fluorescence assay were purchased from Parkard Bioscience. Cell culture medium constituents and phosphate buffered saline (PBS) were purchased from Gibco BRL. Cell Proliferation Kit I (MTT) was purchased from Roche.

HeLa, HepG2, Hep3B and MCF-7 were maintained in a minimum essential medium with Earle's balanced salts (MEM) supplemented with 0.1 mM non-essential amino acids. SUNE-1 and QGY-TR50 were maintained in the RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum and 2 mM *L*-glutamine. Penicillin (100 U/mL) and streptomycin (100 μg/mL) were added to all media. For HeLa cell line, its culture medium was supplemented with 1 mM sodium pyruvate. For QGY-TR50 cell line, it was cultured in culture medium containing 50 nM taxol

for maintaining its normal resistant ability. Cultures were incubated at 37 °C in a 5% CO_2/95% air humidified atmosphere.

Physical measurement

All ^{1}H NMR spectra were recorded on Bruker DPX-300 and -400 NMR spectrometers. FAB and EI mass spectra were recorded on a Finnigan MAT95 mass spectrometer. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Finnigan LCQ spectrometer. Absorption spectra were recorded on a Perkin-Elmer Lambda 900 UV-visible spectrophotometer. Magnetic property of **1a** was performed using Evan's method. Cyclic voltammetry was recorded by using a PAR Potentiostat/Galvanostat Model 273A at scan rate of 100 mV s^{-1} with 0.1 M tetrabutylammonium hexafluorophosphate in acetonitrile as supporting electrolyte at room temperature (after purging the solution with N_2). The working electrode was a glassy carbon (Atomergic Chemetal V25, geometric area of 0.35 cm^2) electrode and the counter-electrode was a platinum gauze. A non-aqueous $Ag/AgNO_3$ (0.1 M in acetonitrile) reference electrode was contained in a separate compartment connected to the solution for measurement via fine sintered glass disks. The ferrocenium/ferrocene was used as internal standard.

X-ray crystallography

Crystals of **1a** were obtained from concentrated acetonitrile solution under N_2 at 0 °C. A red crystal of dimensions 0.3 × 0.2 × 0.05 mm mounted in a glass capillary was used for data collection at 28 °C on a MAR diffractometer with a 300 mm image plate detector using graphite monochromatized Mo-K_α radiation (λ = 0.71073 Å). Data collection was made with 3° oscillation step of φ, 480 seconds exposure time

and scanner distance at 120 mm. 56 images were collected. The images were interpreted and intensities integrated using program DENZO [Z. Otwinowski and W. Minor, In *Processing of X-ray Diffraction Data Collected in Oscillation Mode*, *Methods in Enzymology*, C. W. Carter, Sweet Jr. & R. M., Eds.; Academic Press: 1997; Vol. 276, pp. 307-326]. The structure was solved by direct methods employing SIR-97 program [A. Altomare, M. C. Burla, M. Camalli, G. Cascarano, C. Giacovazzo, A. Guagliardi, A. G. G. Moliterni, G. Polidori, R. Spagna, *J. Appl. Crystl.*, 1998, **32**, 115] on PC. Fe, Cl and many non-H atoms were located according to the direct methods and the successive least-square Fourier cycles. Positions of other non-hydrogen atoms were found after successful refinement by full-matrix least-squares using program SHELXL-97 [G. M. Sheldrick, *SHELX97*. *Programs for Crystal Structure Analysis (Release 97-2)*, University of Goetingen, Germany, 1997]. Two perchlorate anions were located and one of them is disordered. One and a half acetonitrile solvent molecules were located, in which the half acetonitrile located near a special position. For convergence, perchlorate anions were restrained to be normal, i.e. with similar Cl-O and O… O distances.

Crystals of the chloro-analogue of **2**, $[Fe(Py_5\text{-}OH)Cl](CF_3SO_3)$ which was prepared by reacting the ligand of Py_5-OH with $FeCl_2 \bullet 6\ H_2O$ followed by precipitation with $AgCF_3SO_3$, were obtained from slow diffusion of diethyl ether to a solution of $[Fe(Py_5\text{-}OH)Cl](CF_3SO_3)$ in methanol. A yellow crystal of dimensions 0.3 × 0.15 × 0.1 mm mounted in a glass capillary was used for data collection at -20°C on a MAR diffractometer with a 300 mm image plate detector using graphite monochromatized Mo-K_α radiation (λ = 0.71073 Å). Data collection was made with 2° oscillation step of φ, 600 seconds exposure time and scanner distance at 120 mm.

100 images were collected. The images were interpreted and intensities integrated using program DENZO. The structure was solved by direct methods employing SIR-97 program on PC. Fe, Cl, S and many non-H atoms were located according to the direct methods and the successive least-square Fourier cycles. Positions of other non-hydrogen atoms were found after successful refinement by full matrix least-squares using program SHELXL-97 on PC. One CF_3SO_3 anion and one methanol solvent molecule were located.

Stability tests

The UV-vis absorption spectra of all the iron(II) polypyridyl complexes studied in this work were monitored over 6 days in 2 mM of GSH with 1% CH_3CN.

Preparation of iron(II) polypyridine complexes

The synthesis of the iron(II) polypyridine complexes was conducted under N_2 atmosphere using standard Schlenk technique. In general, a mixture of ligand (0.13 mmol) and iron(II) perchlorate hexahydrate (0.2 mmol) in a 50 mL Schlenk tube was degassed and filled with N_2. Freshly distilled and degassed acetonitrile (20 mL) was added to the reaction mixture. The reaction mixture was gently warmed (~ 50 °C) for 12 hr. Red (**1a** and **1b**) and yellow (**2**) solutions were obtained at the end of the reaction, which were reduced to 5 mL under reduced pressure. Crystals were obtained after keeping at 0°C for 4 days under N_2 atmosphere.

$[Fe(H\text{-}qpy)(CH_3CN)_2](ClO_4)_2$ (1a): Yield: 75%. UV-vis (CH_3CN): λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}): 292 (19500, sh), 311 (27300), 355 (11100), 347 (10700), 534 (630, broad). ^{1}H NMR (400 MHz, CD_3CN): δ_H –0.18, 0.52, 3.49, 33.74, 43.27, 53.61,

53.78, 75.04. ESI-MS (+ve): *m/z* 242.0 $[Fe(H\text{-}qpy)(CH_3CN)]^{2+}$ and 221.5 $[Fe(H\text{-}qpy)]^{2+}$. Anal. Calcd. for $C_{29}N_7H_{23}Cl_2O_8Fe$ (%): H, 3.20; C, 48.09; N, 13.54. Found: H, 3.13; C, 48.36; N, 13.25.

$[Fe(Ph\text{-}qpy)(CH_3CN)_2](ClO_4)_2$ (1b): Yield: 75 %. UV-vis (CH_3CN): λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}): 292 (43100), 311 (27700, sh), 353 (13400, sh), 420 (2090), 567 (880, broad). ESI-MS (+ve): *m/z* 317.7 $[Fe(Ph\text{-}qpy)(CH_3CN)]^{2+}$ and 297.5 $[Fe(Ph\text{-}qpy)]^{2+}$. Anal. Calcd. for $C_{41}N_7H_{31}Cl_2O_8Fe$ (%): H, 3.56; C, 56.19; N, 11.19. Found: H, 3.52; C, 56.70; N, 11.42.

$[Fe(Py_5\text{-}OH)(CH_3CN)](ClO_4)_2$ (2): Yield: 60%. UV-vis (CH_3CN): λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}): 256 (32800), 345 (26000), 412 (32800). ESI-MS (+ve): *m/z* 271.8 $[Fe(Py_5\text{-}OH)(CH_3CN)]^{2+}$. Anal. Calcd. for $C_{29}N_6O_{10}H_{25}FeCl_2$ (%): H, 3.39; C, 46.80; N, 11.29 . Found: H, 3.52; C, 46.70; N, 11.52.

Cytotoxicity tests

1×10^4 cells/well were seeded in 96-well flat bottomed microtitre plates with supplemented culture medium (100 μL/well) and were incubated at 37 °C with 5% CO_2/95% air for 48 hr. After further incubation for 48 hr, fresh supplemented culture medium was replaced and complex-containing media (100 μL with < 1% CH_3CN, final complex concentration 100 μM) was added. Serial dilution resulted in wells containing a wide range concentration of complex-containing media. Microtitre plates were then incubated at 37 °C with 5% CO_2/95% air for 48 hr depending on the drug treated. All cytotoxicity tests were run parallel with a set of negative control, cells without addition of complex, and also a set of positive control using cisplatin as cytotoxic agent for comparison. At the end of incubation, cytotoxicity was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

10 μL of MTT solution (5 mg MTT/mL in 1 × PBS) was added to each well, and was then incubated for further 4 hr. Then 100 μL solubilization buffer (10% SDS in 0.01 M HCl) was added to each well in order to lysis the cells and solubilize the blue formazan complex formed. Further incubation of the microtitre in dark overnight was required before taking measurement. Formation of formazan was measured using a microtitre plate reader (Perkin-Elmer Fusion^{TM} α-FP) using an absorbance 580 nm filter. IC_{50} of the complex was determined based on the percentage of cell survival in a dose-dependence manner relative to the negative control.

Flow cytometric studies of the 1a complex-induced apoptosis and cell cycle evaluation

Flow cytometric analysis was performed with a Coulter EPICS flow cytometer (Coulter, Miami, FL) equipped with 480 long, 525 band and 625 long pass mirrors. Samples were excited by 15 mW air-cool argon convergent laser at 488 nm. Fluorescence signals were manipulated with Coulter Elite 4.0 software (Coulter) and were analyzed by Winlist 1.04 and Modfit 5.11 software (Verity Software House, Topsham, ME).

For the apoptosis studies, HeLa cells were cultured in 60 mm plates (4×10^5 cells/well) with 5 mL of supplemented medium. After incubation for 24 hrs, complex **1a** with concentration equals to its IC_{40} value was added and the plates were then further incubated for 6, 12, 18 and 24 hr. Staurosporine streptomyces sp. (Calbiochem) was used as positive control. At the end of each incubation period, cells were trypsinized, washed with PBS and stained with Annexin-V/propidium iodide following the procedures given by the Annexin-V-Fluos Staining Kit user's manual

provided by Roche.

For cell cycle evaluation, HeLa cells were cultured in 60 mm plates (4×10^5 cells/well) with 5 mL of supplemented medium. After incubation for 24 hr, complex **1a** with concentration equals to its IC_{40} value was added with fresh supplemented media and the plates were further incubated for 6, 12, 18 and 24 hr. Staurosporine streptomyces sp. (Calbiochem) was used as positive control. At the end of each incubation period, cells were trypsinzed and washed with PBS. Cells were then fixed with 50 % EtOH in 1 × PBS and kept at -80°C overnight before analysis. Before staining, cells were spined at 3000 rpm for 5 min and the cell pellet was washed with 1× PBS. The cells were fixed and stained with DNA-Pre COULTER® reagent kit (Beckman Coulter) and were ready for analysis.

Cleavage of supercoiled plasmid DNA

pCR 2.1 supercoiled plasmid DNA was prepared by maxi preparation according to standard protocol. The purity of supercoiled plasmid DNA was determined by running the plasmid DNA on a 1.2% agarose gel to determine the percentage of nicked and linear DNA present. The reactions were initiated by addition of metal complex (0.6 μM) to a 10 mM Tris buffer solution (pH 7.5) of supercoiled plasmid DNA (0.06 μg/μL) at 37 °C in the presence of air without the addition of reductant. At indicated time interval, 10 μL of sample was taken from reaction mixture and was quenched by loading dye (2 μL, consisting of 0.04% bromophenol blue, 0.04% xylene cyanol and 5% glycerol) and kept at 0 °C immediately. The samples were loaded onto a 1.2% agarose gel running at 70 V in 1 × TAE buffer for 3 hr and stained with ethidium bromide afterwards. The image was visualized and photos were taken

by Olympus C-4040Z digital camera. Bands on the film were quantified using the software program AlphaEaseFC^{TM}.

Determination of reactive oxygen species (ROS) formation *in vitro* by dihyrodrhodamine-123

Intracellular reactive oxygen species generation triggered by iron(II) complexes *in vitro* was measured by oxidation of non-fluorescent dihydrorhodamine-123 (DHRh-123) to green fluorescence rhodamine-123^{+} (Rh-123^{+}) as described by Yang and co-workers [*Environ. Health Perspect*., 1997, **105**, 712]. The stock solution of DHRh-123 (1×10^{-2} M) was prepared in DMSO and purged with argon prior to storage in the dark at –80 °C. Cells (8000 cells/well) were cultured in 96-well black-bottomed plate 24 hr before performing the experiment. Cells were then washed once with 1 × Hank's balanced salt solution (HBSS) and then were incubated for 0, 60, 120 and 180 min in the presence and absence of iron(II) complexes, with 5 μM/L DHRh-123 (Molecular Probes). At the end of incubation period, the cells were washed twice with HBSS. The intensity of Rh-123^{+}, formed by the interaction of DHRh-123 with intracellular oxidants was subsequently evaluated using a Perkin-Elmer spectrofluorometer Fusion^{TM} α-FP using excitation and emission wavelengths of 485 and 530 nm, respectively. The ratio of intracellular production of oxidants in response to iron(II) complex treatment was evaluated based on the relative fluorescence intensity relative to the untreated control.

Figures and tables

Fig. S1 ORTEP drawing of **1a** cation. Hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 30% probability level. Selected interatomic distances (Å): Fe-N(1) 2.351(10), Fe-N(2) 2.254(10), Fe-N(3) 2.255(10), Fe-N(4) 2.271(11), Fe-N(5) 2.284(11), Fe-N(6) 2.183(12), Fe-N(7) 2.184(12).

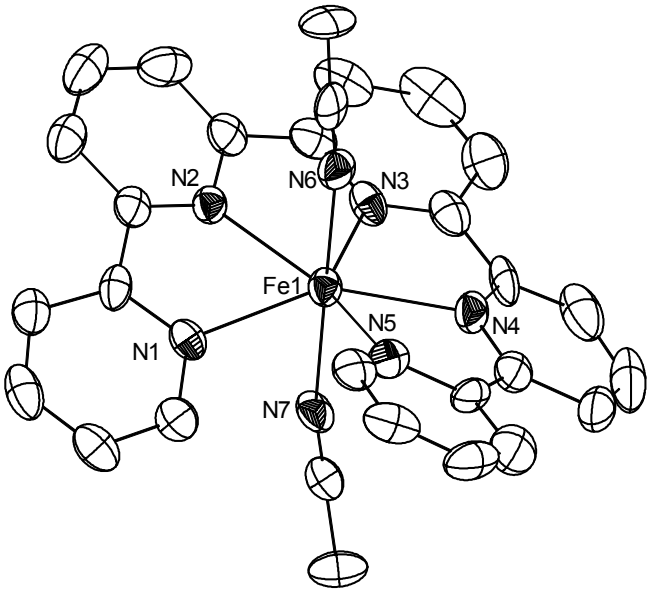

Fig. S2 ORTEP drawing of **1b** cation. Hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 30% probability level. Selected interatomic distances (Å): Fe-N(1) 2.353(17), Fe-N(2) 2.205(17), Fe-N(3) 2.256(17), Fe-N(4) 2.270(17), Fe-N(5) 2.327(19), Fe-N(6) 2.314(19), Fe-N(7) 2.33(2).

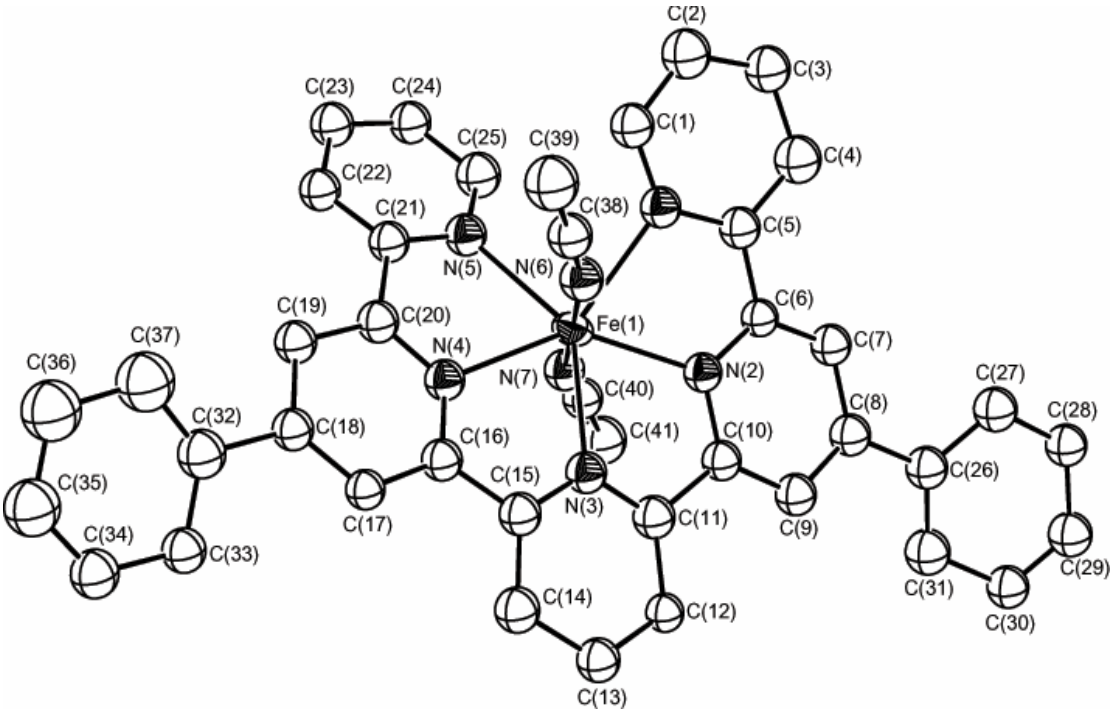

Fig. S3 ORTEP drawing of the chloro-analogue of **2** cation. Hydrogen atoms are omitted for clarity. Thermal ellipsoids are drawn at the 30% probability level. Selected interatomic distances (Å): Fe-N(1) 2.212(5), Fe-N(2) 2.197(5), Fe-N(3) 2.196(5), Fe-N(4) 2.187(5), Fe-N(5) 2.191(5), Fe-Cl(1) 2.3878(19).

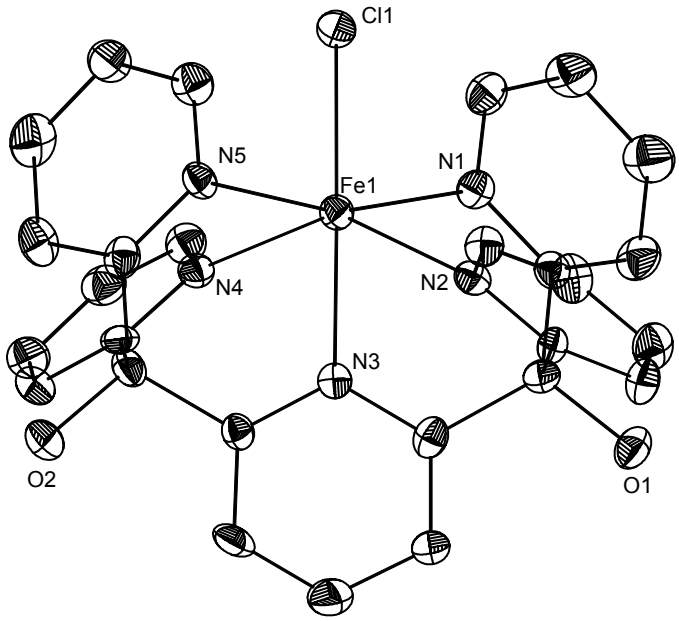

Fig. S4 Cleavage of pCR 2.1 supercoiled plasmid DNA (0.06 μg/μL) with iron(II) complexes at 37 °C in a concentration and time dependent manner.

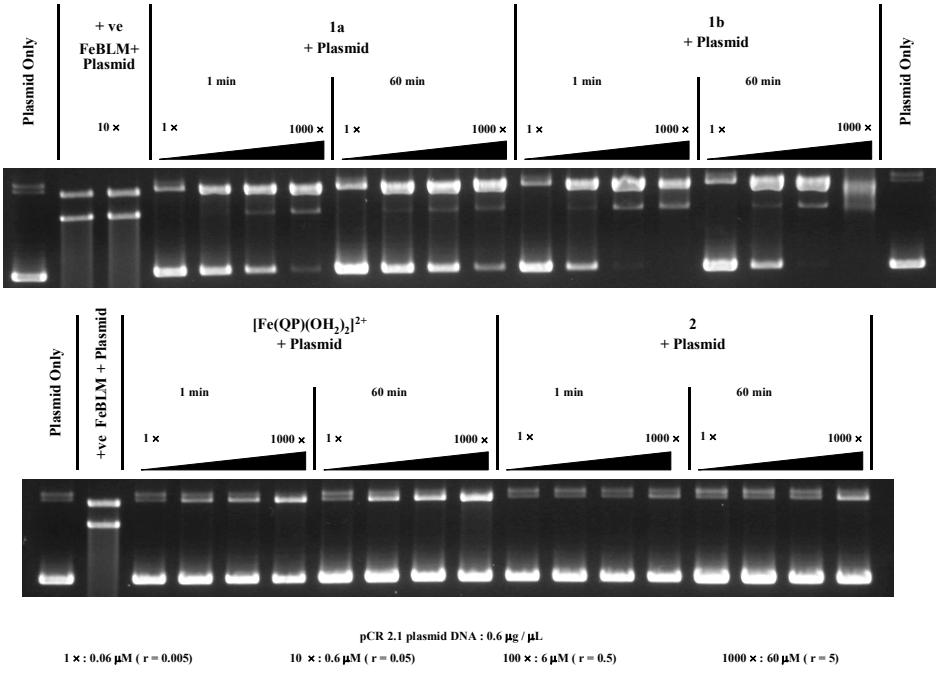

Fig. S5 Cytotoxicity profiles of **1a** toward carcinoma cell lines HeLa, MCF-7, HepG2, Hep3B, SUNE-1, QGY-TR50 and normal human lung fibroblast cell line CCD-19Lu. Graphs show the percentage growth compared to untreated cells upon incubation.

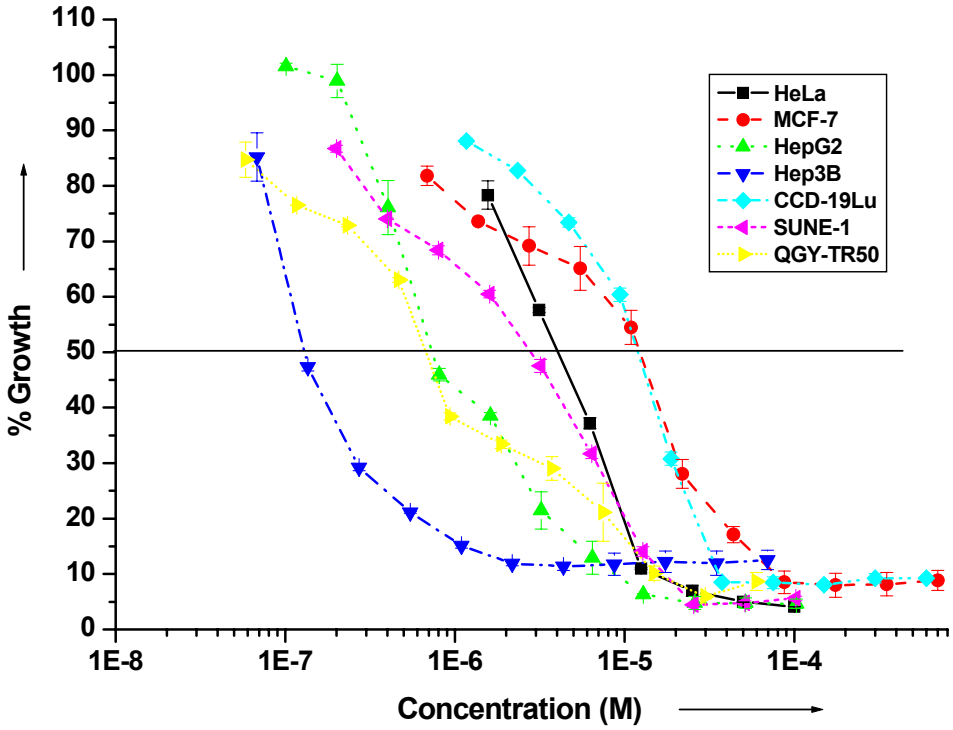

Table S1 Cleavage of the pCR 2.1 supercoiled plasmid DNA (0.06 μg / μL) with t = 10 s. Significant DNA cleavage was observed for **1a**, **1b** (>50%) and Fe(BLM) (≈ 100%). Fe(BLM) (0.6 μM) afforded both nicked (47%) and linear (53%) DNA. **1a** and **1b** (0.6 μM) mainly afforded nicked DNA (64% and 55% respectively), typical for single-strand cleavage agent.

Reagent	Form I	Form II	Form III
control	100	0	0
1a (0.6 μM)	32	64	4
1a (6 μM)	24	71	5
1b (0.6 μM)	45	55	0
1b (6 μM)	37	63	0
2 (0.6 μM)	99	1	0
2 (6 μM)	98	2	0
Fe(BLM) (0.6 μM)	0	47	53

Table S2 Cell cycle evaluation of **1a**-treated HeLa cells.

	Cell cycle (%)			
Time (hr)	G0/G1	G2/M	S	G2/G1
0	52.7 ± 2.7	37.6 ± 4.6	9.6 ± 1.8	1.72 ± 0.02
6	45.60 ± 0.52	27.91 ± 0.77	26.50 ± 0.25	1.71 ± 0.02
12	42.4 ± 5.7	42.3 ± 8.9	29.98 ± 0.10	1.68 ± 0.08
18	18.9 ± 1.0	26.6 ± 1.4	54.58 ± 0.41	1.88 ± 0.01
24	5.79 ± 0.53	14.7 ± 1.6	83.46 ± 0.98	2.99 ± 0.02