Metal binding cationic imidazopyridine derivative for antitumor activity and cellular imaging†

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Electronic Supplementary Information (ESI)†

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Experimental

DNA binding experiment

DNA binding experiments were carried out in Tris-HCl/NaCl buffer (5 mM Tris-HCl, 5 mM NaCl, pH 7.2) using aqueous solutions of compound **1**. Calf thymus (CT) DNA in Tris-HCl buffer medium gave a ratio of the UV absorbance at 260 and 280 nm of ca.1.9:1 suggesting the DNA apparently free from protein. The concentration of DNA was measured from its absorption intensity at 260 nm with known molar absorption coefficient value of 6600 M^{-1} cm^{-1}.[1] UV-visible absorption titration experiments were carried out using calf thymus (CT) DNA (ca. 250 μM NP) in Tris-HCl buffer medium with a compound **1** concentration of 10 μM. Due correction was made for the absorbance of CT DNA itself. Samples were kept for equilibration before recording each spectrum. The binding constant values (K_b) were determined using McGhee-von Hippel method using the expression of Bard and co-workers:

$\Delta\varepsilon_{af}/\Delta\varepsilon_{bf} = (b - (b^2 - 2K_b^2C_t[DNA]/s)^{1/2})/2K_b$, ... (1)

$b = 1 + K_bC_t + K_b[DNA]/2s$,

where K_b is the microscopic binding constant for each site, C_b is the concentration of the DNA bound ligand, C_t is the total concentration of the compound, and s is the site size (in base pairs) of the compound interacting with the CT DNA.[2,3] The non-linear least-squares analysis was done using Origin Lab, version 6.1.

DNA denaturation experiments were carried out by monitoring the absorbance of CT DNA (260 nm) in the temperature range of 40 – 90 °C in the absence and presence of compound **1** in a 20:1 molar ratio of the CT DNA and the compound **1** with a ramp rate of 0.5 °C min^{-1} in 5 mM phosphate buffer medium (pH, 6.85) using Cary 300 bio UV-Vis spectrometer with Cary temperature controller.[4] Viscommetric titration experiments were carried out using Schott Gerate AVS 310 automated viscometer attached with constant temperature bath at 37 °C to evaluate the nature of the interaction of the compound to the CT DNA. The concentration of CT DNA stock solution was 150 μM (NP) in 5 mM Tris-HCl buffer. The flow times were measured with an automated timer. The data were presented as relative specific viscosity of DNA, $(\eta/\eta_0)^{1/3}$ versus [compound]/[DNA], where η is the viscosity of CT DNA in the presence of 1 and η_0 is that of CT DNA alone.[5] The viscosity values were calculated from the observed flow time of CT DNA containing solutions (t) duly corrected for that of the buffer alone (t_0), $\eta = (t-t_0)$. The nature of

DNA binding ability of the small molecules was based on an increase of viscosity of the DNA solution when small molecule binds to the DNA.

Isothermal titration calorimetric (ITC) experiments were performed using Nano-Isothermal Titration Calorimeter III, Model CSC 5300. In a typical isothermal calorimetric titration experiment, 1.0 ml solution of CT-DNA in 5 mM Tris-HCl/25 mM NaCl buffer was placed in the sample cell of the calorimeter and equilibrated to 20 °C. A 100 μM CT DNA was used for the titration against 2 mM of compound **1**. The compound solution which was placed in the injection syringe of 250 μL was injected into the sample cell in 31 injections with 8 μL per injection. Control titrations of the compound into the buffer were also performed in order to determine background heat of dilution that was found to be insignificant. The data were fitted to single set of identical binding sites model using the Bind Works, version 3.1.11 software.[6]

DNA photocleavage experiment

The cleavage of supercoiled (SC) pUC19 DNA (1 μL, 30 μM, 2686 base pairs) by compound **1** (2 μL) was studied by agarose gel electrophoresis in 50 mM Tris-HCl buffer (pH = 7.2) containing 50 mM NaCl.[7] The photo-induced DNA cleavage reactions were carried out under illuminated conditions using UV-A lamp of 365 nm (6 W, sample area of illumination: 45 mm^2). Eppendorf vials were used for UV-A light experiments in a dark room at 25 ºC using SC DNA (1 μL, 30 μM) in 50 mM Tris-(hydroxymethyl)methane-HCl (Tris-HCl) buffer (pH 7.2) containing 50 mM NaCl and the compound (2 μL in DMF) with varied concentrations. The concentration of **1** in water or the additives in buffer corresponded to the quantity in 2 μL stock solution used prior to dilution to the 20 μL final volume using Tris-HCl buffer. The solution path-length used for illumination in the sample vial was ~5 mm. After the photo-exposure, the sample was incubated for one hour at 37 ºC, followed by its addition to the loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol (2 μL), and the solution was finally loaded on 0.8% agarose gel containing 1.0 μg mL^{-1} ethidium bromide. Electrophoresis was carried out in a dark room for 2 h at 45 V in TAE (Tris-acetate-EDTA) buffer. The bands were visualized by UV light and photographed. The extent of cleavage of SC DNA was determined by measuring the intensities of the bands using a UVITECH Gel Documentation System. Due corrections were made for the low level of nicked circular (NC) form present in the original

supercoiled (SC) DNA sample and for the low affinity of EB binding to SC compared to NC and linear forms of DNA.[8] The error range observed in determining %NC form from the gel electrophoresis experiments was ±3-5%. Different additives were added to the SC DNA for mechanistic investigations in presence of **1** prior to light exposure.

Cell viability assay

The cellular toxicity of the cationic compound **1** in the presence or absence of the metal ions, viz. $FeSO_4•7H_2O$, $Cu(NO_3)_2•3H_2O$ or $ZnSO_4•7H_2O$ was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay based on the ability of mitochondrial dehydrogenases in the viable cells to cleave the tetrazolium rings of MTT, forming dark blue membrane impermeable crystals of formazan that can be quantified at 595 nm on detergent solubulization.[7,9,10] The amount of the formazan product formed gives a measure of the viable cells. Approximately, 15000 HeLa cells (human cervical carcinoma cell line), HepG2 (human liver carcinoma cell line) and Jurkat (human T-cell leukemia cell line) were plated in a 96-well culture plate in DMEM (for HeLa & HepG2) or RPMI-1640 (for Jurkat) supplemented with 10% fetal bovine serum. After 24 h of incubation at 37 °C in a CO_2 incubator, different concentrations of compound **1** (0.1, 0.5, 1, 2, 5, 10 and 20 μM) in the presence or absence of metal ion Fe^{2+}, Cu^{2+} or Zn^{2+} (50 μM) were added to the cells, and the incubation was continued for 12 h in the dark. After incubation, the medium in HepG2 and HeLa cells plates was replaced with PBS and photoirradiated with UV-A light of 365 nm (fluence rate: 610 μW cm^{-2} for 15 min) to provide a total dose of 0.55 *J* cm^{-2}. Post irradiation, PBS was replaced with DMEM-FBS, and incubation was continued for a further 36 h in the dark. After 48 h, 20 μL of 5 mg mL^{-1} of MTT was added to each well and incubated for an additional 3 h. The culture medium was discarded, and 200 μL of DMSO was added to dissolve the formazan crystals. The absorbance at 595 nm was determined using a BIORAD ELISA plate reader. The cytotoxicity of the test compound was measured as the percentage ratio of the absorbance of the treated cells to the untreated controls. The IC_{50} values were determined by nonlinear regression analysis (Graph Pad Prism).

Nuclear staining

The changes in chromatin organization following photoexposure after treatment with **1** in the presence or absence of metal ion Fe^{2+}, Cu^{2+} or Zn^{2+} (50 μM) were determined microscopically by assessing staining with Hoechst 33258 and an acridine orange/ethidium bromide (AO/EB) dual

stain. Hoechst staining was performed as described in the literature.[11] Briefly, around 50,000 cells were allowed to adhere overnight on 25 mm coverslip placed in each well of 24 well plate and the control and the cells treated with 1 (4 μM) in presence or absence of the metal ions for 12 h in the dark, followed by irradiation with UV-A light of 365 nm (fluence rate: 610 μW cm^{-2} for 15 min) to provide a total dose of 0.55 *J* cm^{-2}. The cells were fixed with 4% (v/v) paraformaldehyde in PBS (pH-7.4) for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and stained with Hoechst 33258 (1 mg mL^{-1} in PBS) for 5 min. After being washed twice with PBS and were examined by fluorescence microscopy (360/40 nm excitation and 460/50 nm emission filters). The apoptotic cells were identified by the presence of highly condensed or fragmented nuclei. The protocol for AO/EB that was used was derived from the reported ones.[12] HeLa cells were cultured and treated as described above. The cells were then allowed to recover for 1 h or washed thrice with PBS and stained with an AO/EB mixture (1:1, 10 μM) for 15 min and observed at 40x magnification with a fluorescence microscope using 485/20 nm excitation and 535/40 nm emission filter sets.

References

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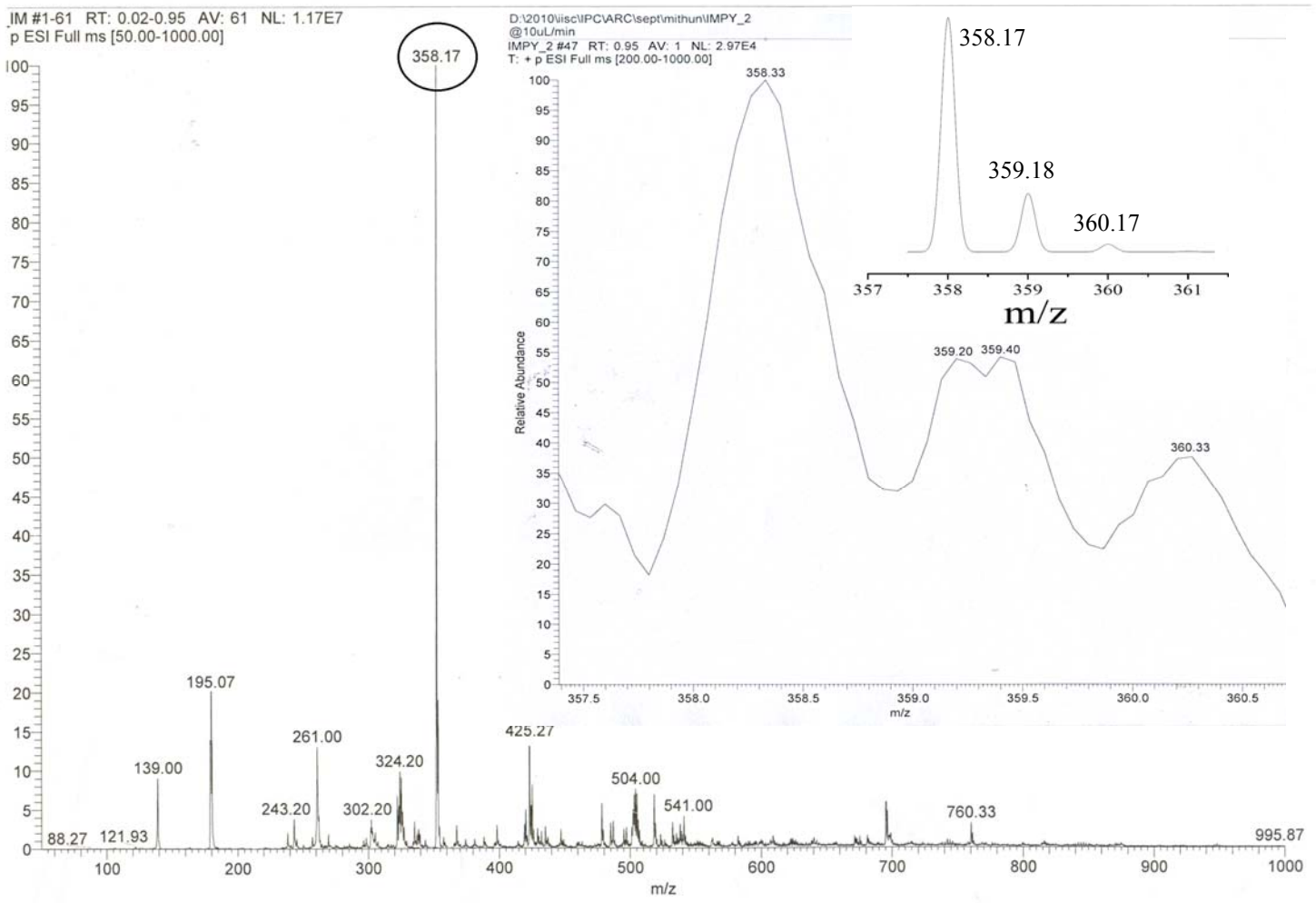


Fig. S1. ESI-MS spectrum of **1** in H_2O-MeOH showing the parent ion peak at m/z 358.17 $[M-(PF_6^-)]^+$. The peak at m/z 195.07 corresponds to the imidazopyridine nucleus resulting from the degradation of the compound **1** under the ESI MS record condition. The inset shows the isotopic distribution of the peak at m/z 358.17 along with the simulated one. The simulation was performed using Molecular Weight Calculator.

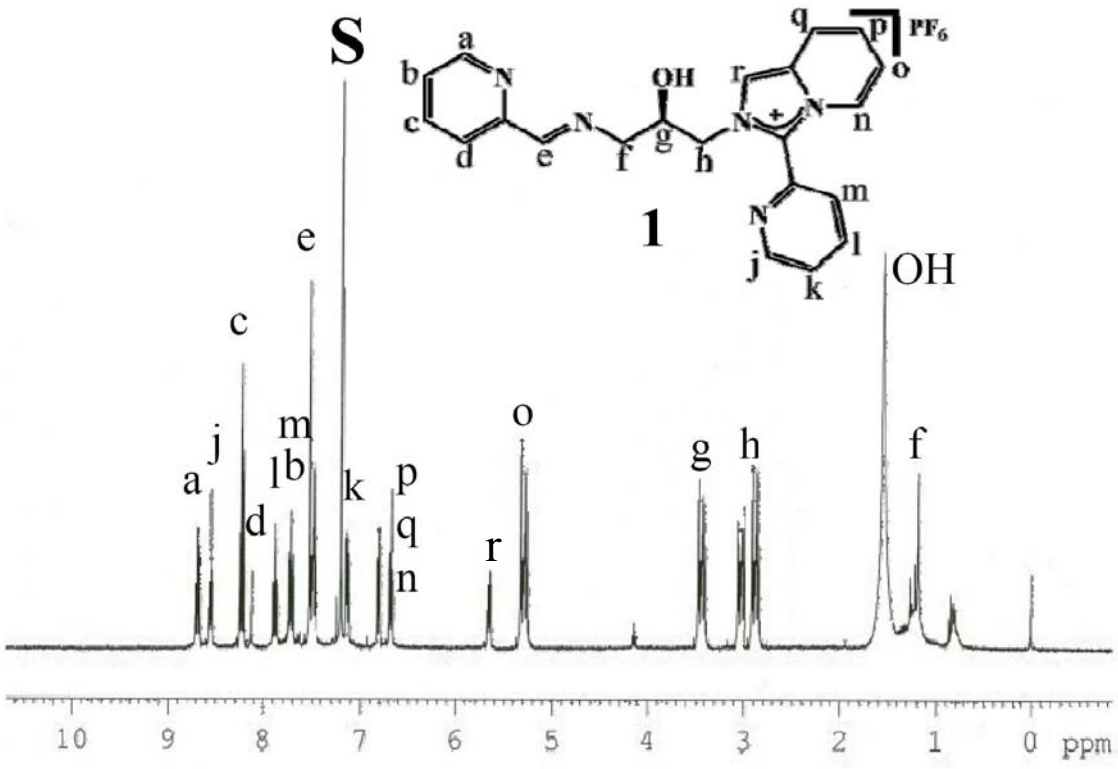


Fig. S2. ^{1}H NMR spectrum of **1** in $CDCl_3$ with TMS as the standard (S = solvent peak). The protons are assigned and the assignments are shown in the inset drawing.

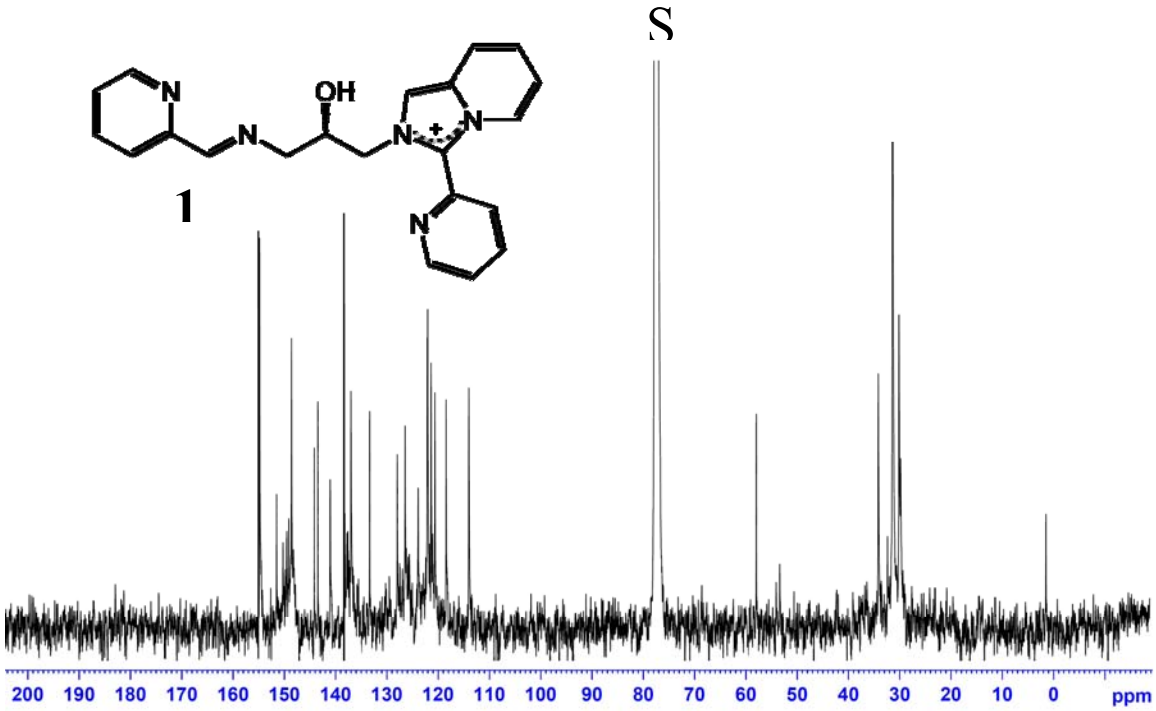


Fig. S3. ^{13}C NMR spectrum of **1** in $CDCl_3$ with TMS as the standard (S = solvent peak). Total 21 peaks in the spectrum correspond to 21 carbon atoms of the compound **1**. The peaks corresponding to aliphatic carbons appear within δ 25-60 ppm. Aromatic carbons have been assigned in the region of δ 110-155 ppm.

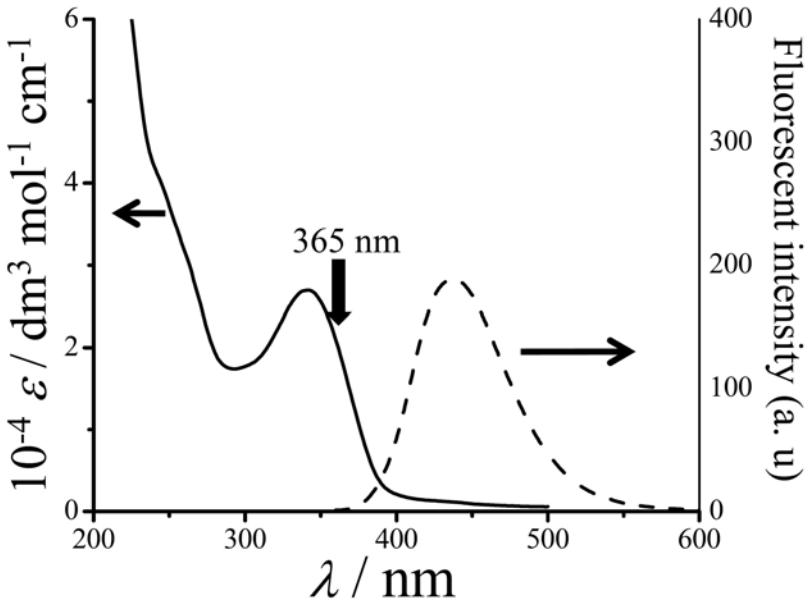


Fig. S4. UV-visible absorption spectrum of **1** in Tris-HCl buffer (pH 7.2). The dashed-line represents the emission spectrum of **1** in Tris-HCl buffer medium (pH 7.2). The excitation wavelength used is 342 nm. The wavelength (UV-A light, 365 nm) used for photocleavage of DNA is also shown.

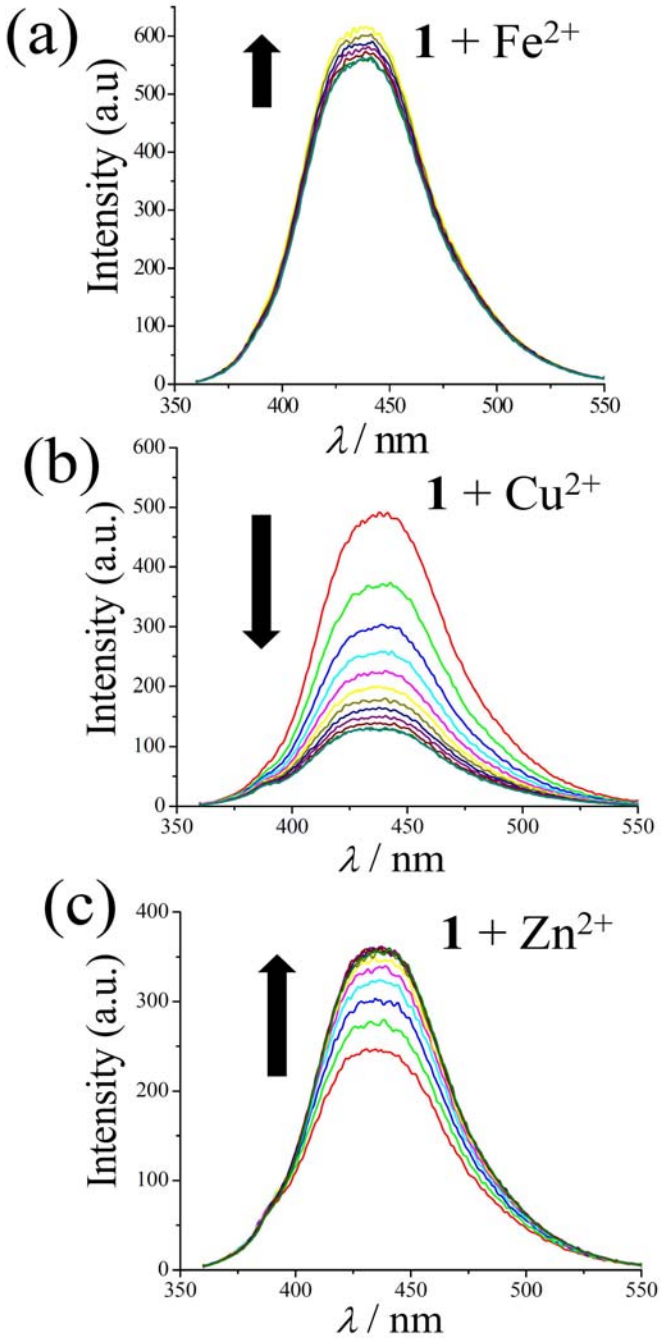


Fig. S5. Effect of increasing concentration of Fe^{2+} (a), Cu^{2+} (b) and Zn^{2+} (c) on the fluorescence intensity of the compound **1** (10 μM) in Tris-HCl buffer medium. Fe^{2+} addition shows marginal increase, Cu^{2+} shows significant decrease and Zn^{2+} shows significant increase in fluorescence intensity.

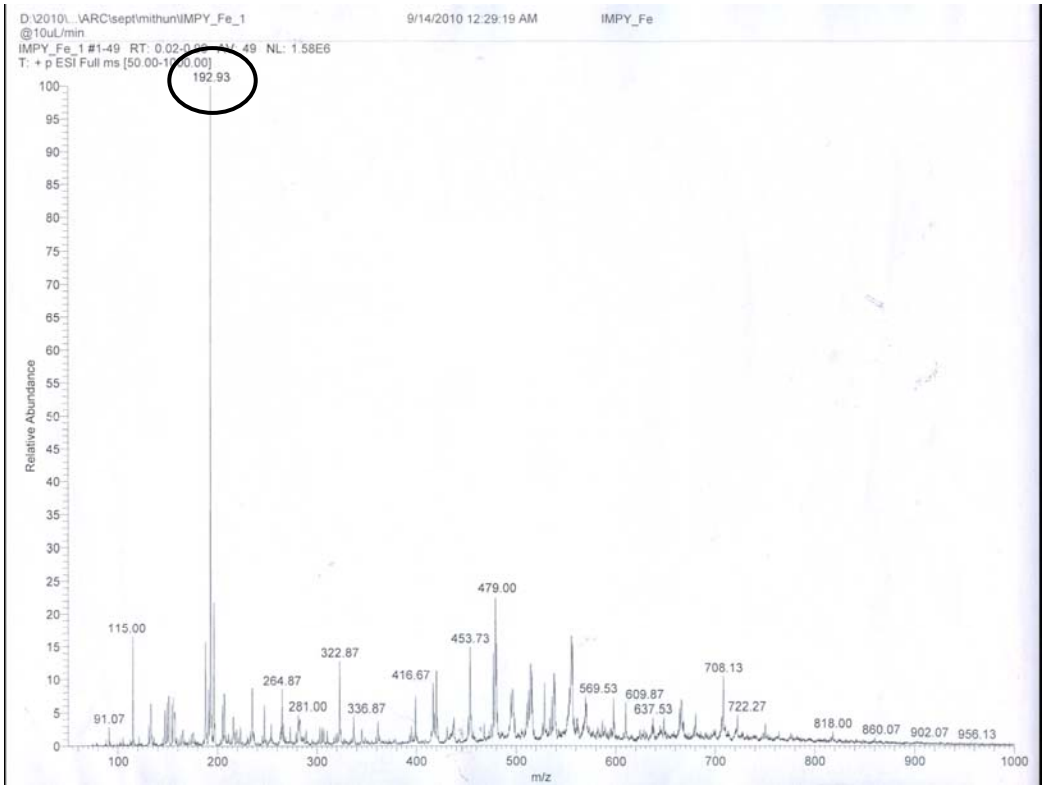


Fig. S6. ESI-MS spectrum of **1** in the presence of Fe^{2+} (1: 12 molar ratio) in H_2O-MeOH showing the parent ion peak at m/z 192.93. The peak corresponds to 1:2 complex $[Fe(\mathbf{1})_2]^{4+}$.

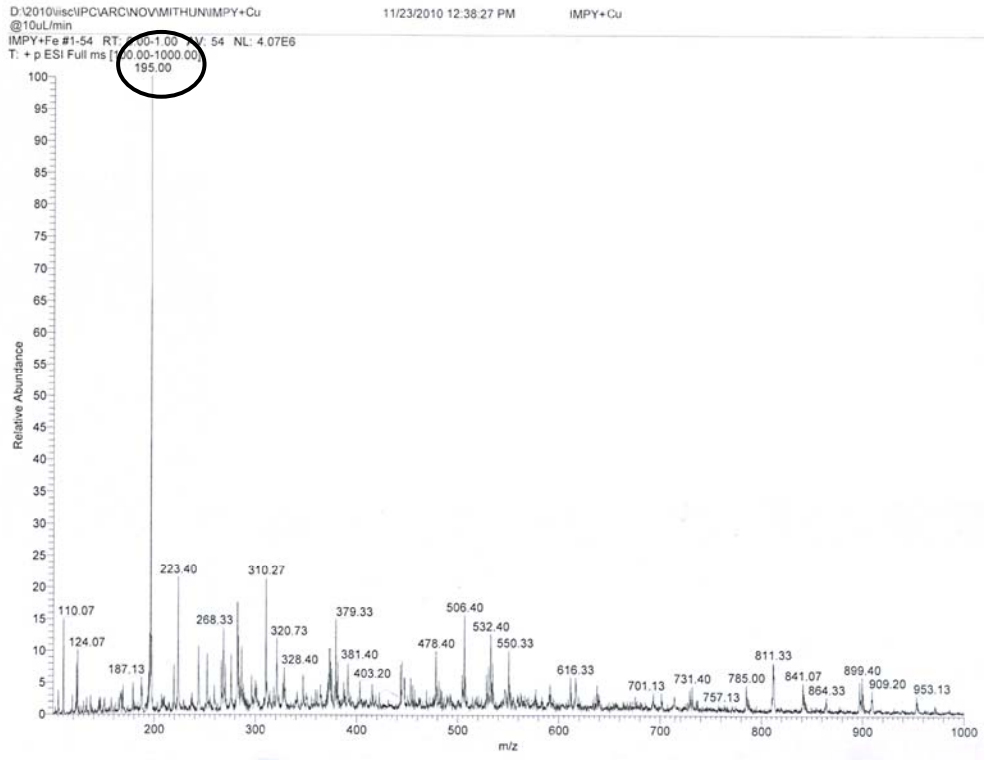


Fig. S7. ESI-MS spectrum of **1** in the presence of Cu^{2+} (1: 12 molar ratio) in H_2O-MeOH showing the parent ion peak at m/z 195.00. The peak corresponds to 1:2 complex $[Cu(\mathbf{1})_2]^{4+}$.

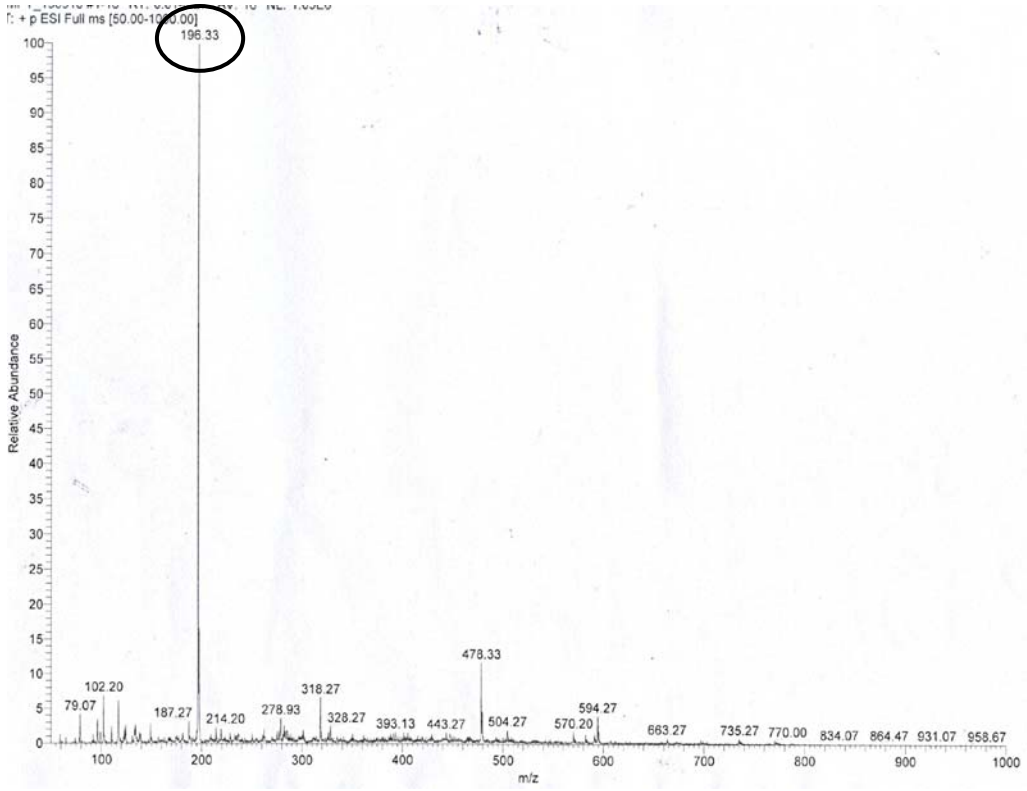


Fig. S8. ESI-MS spectrum of **1** in the presence of Zn^{2+} (1: 12 molar ratio) in H_2O-MeOH showing the parent ion peak at m/z 196.33. The peak corresponds to 1:2 complex $[Zn(\mathbf{1})_2]^{4+}$.

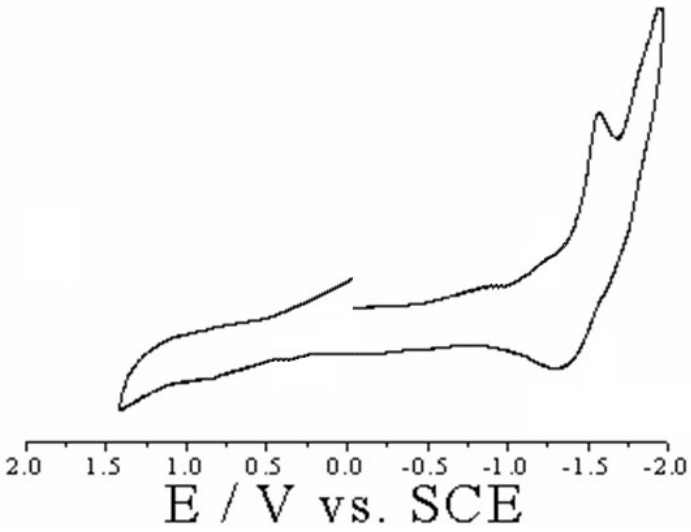


Fig. S9. Cyclic voltammogram of **1** in H_2O-0.1M KCl at a scan rate of 50 mV s^{-1} showing a cathodic peak.

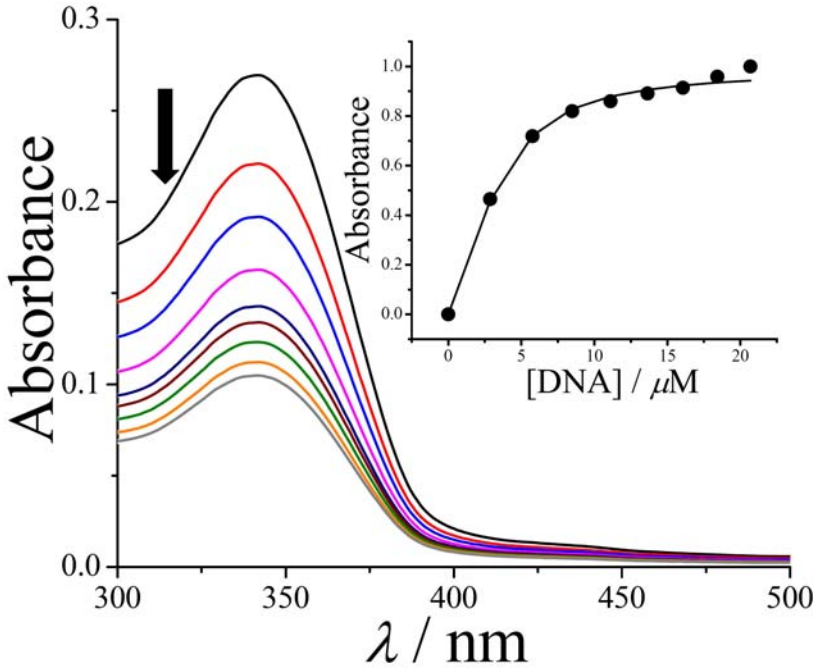


Fig. S10. Absorption spectral traces showing a decrease in absorption intensity on gradual addition of CT-DNA (250 μM) in aliquots to the solution of **1** (10 μM) in 5 mM Tris-HCl buffer (*p*H, 7.2) at 25°C. The inset shows the plot of $\Delta\varepsilon_{af}/\Delta\varepsilon_{bf}$ *vs.* [DNA].

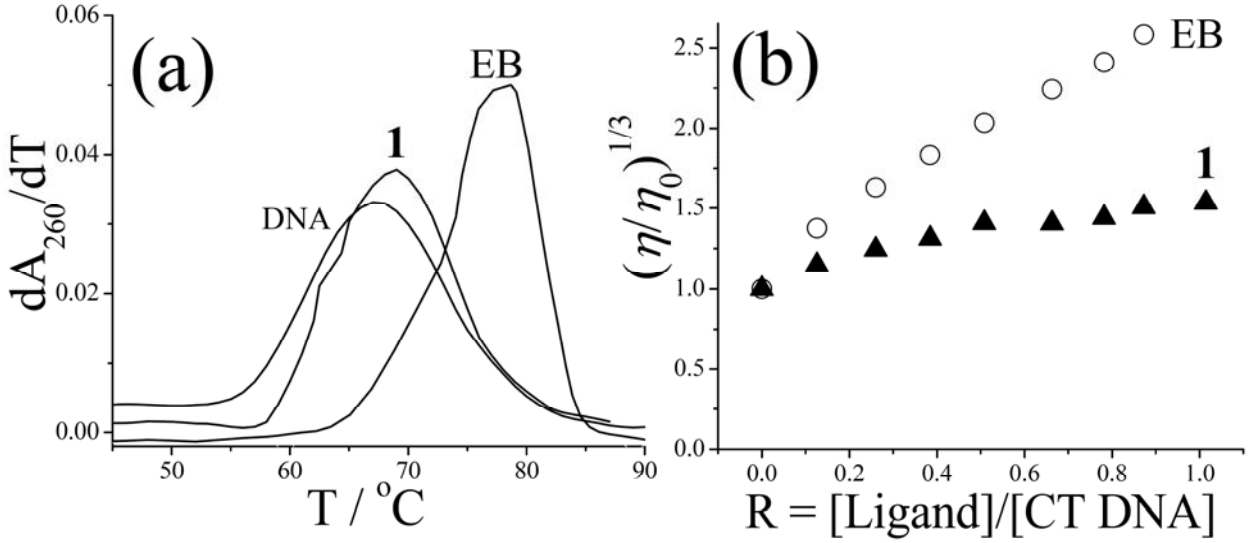


Fig. S11. (a) DNA melting curves for CT-DNA in the absence and presence of compound **1** and ethidium bromide (EB) in phosphate buffer (pH 6.8), [DNA]/[ligand] = 20:1. (b) Effect of increasing the quantity of **1** (▲) and EB (○) on the relative viscosities of CT-DNA at 37.0 (± 0.1) ºC in 5 mM Tris-HCl buffer (*p*H, 7.2) ([DNA] = 150 μM and R = [ligand]/[DNA]).

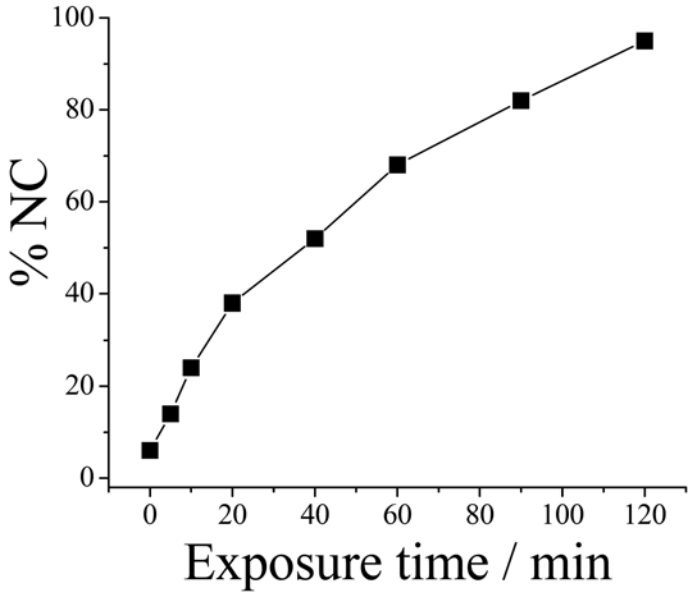


Fig. S12. Extent of photo-induced DNA cleavage activity of **1** (10 μM) at different exposure times (0, 5, 10, 20, 30, 60, 90 and 120 min) in UV-A light of 365 nm (6W).

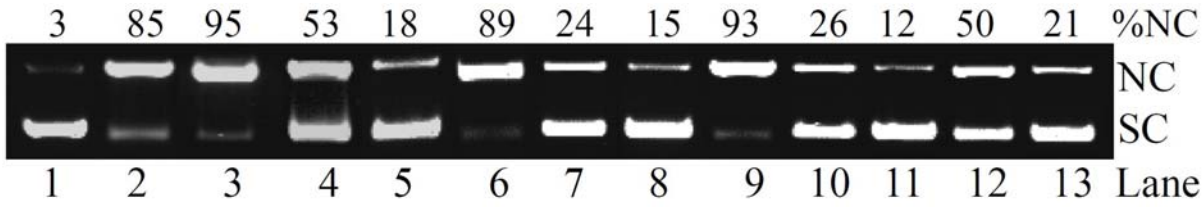


Fig. S13. Gel electrophoresis diagram showing the cleavage of SC pUC19 DNA (0.2 μg, 30 μM) by compound **1** in UV-A light of 365 nm (6 W) in 50 mM Tris-HCl/NaCl buffer (*p*H, 7.2) for 2 h exposure time: lane-1, DNA control; lane-2, DNA + Fe^{2+} + **1**; lane-3, DNA + Cu^{2+} + **1**; lane-4, DNA + Zn^{2+} + **1**; lane-5, DNA + distamycin-A + Fe^{2+} + **1**; lane-6, DNA + methyl green + Fe^{2+} + **1**; lane-7, DNA + Fe^{2+} + **1** (in Argon); lane-8, DNA + distamycin-A + Cu^{2+} + **1**; lane-9, DNA + methyl green + Cu^{2+} + **1**; lane-10, DNA + Cu^{2+} + **1** (in Argon); lane-11, DNA + distamycin-A + Zn^{2+} + **1**; lane-12, DNA + methyl green + Zn^{2+} + **1**; lane-13, DNA + Zn^{2+} + **1** (in Argon) ([Fe^{2+}] = [Cu^{2+}] = [Zn^{2+}] = 50 μM; [**1**] = 1 μM; [distamycin-A] = [methyl green] = 100 μM).

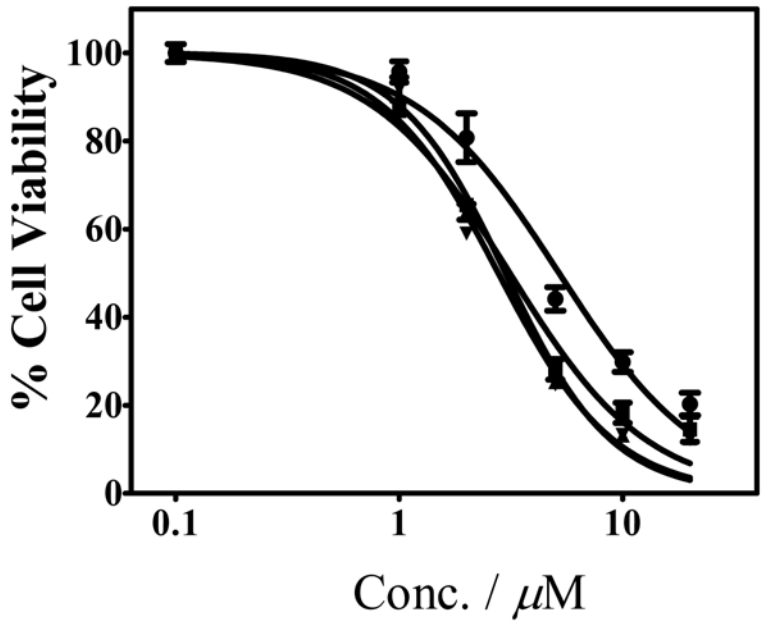


Fig. S14. Cytotoxicity of **1** in Jurkat cells cells: treated with **1 (●)**; treated with **1** in the presence of Fe^{2+} (■); treated with **1** in the presence of Cu^{2+} (▲) and treated with **1** in the presence of Zn^{2+} (▼) ($[Fe^{2+}] = [Cu^{2+}] = [Zn^{2+}] = 50$ μM).

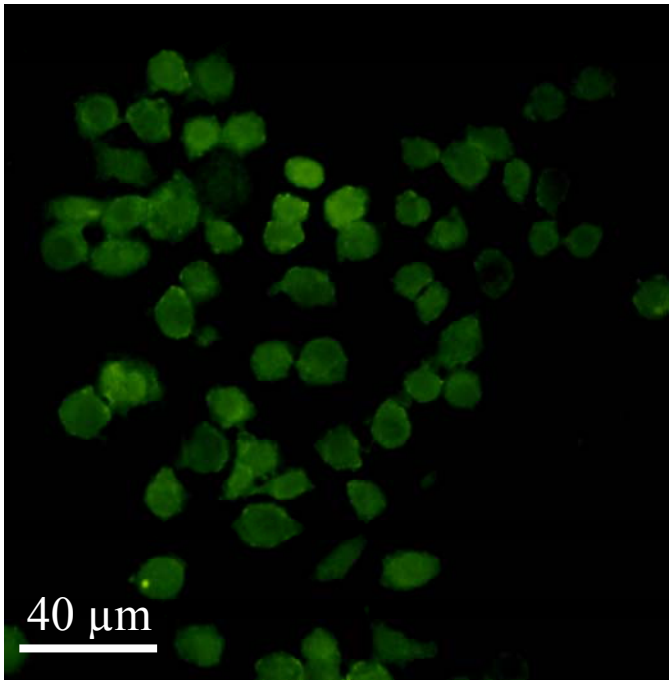


Fig. S15. HeLa cells showing DCF fluorescence when treated with 100 μM H_2O_2 suggesting generation of ROS. HeLa cells treated with H_2O_2 are taken as positive control.

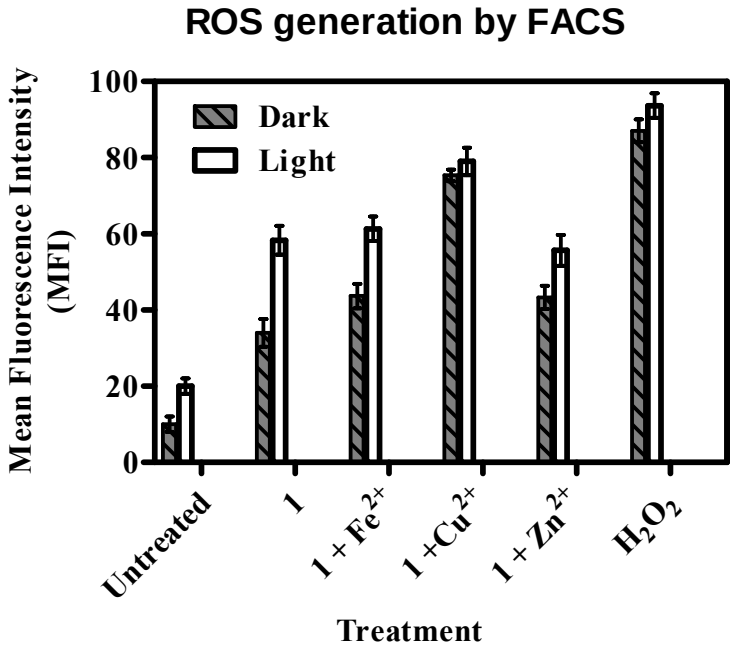
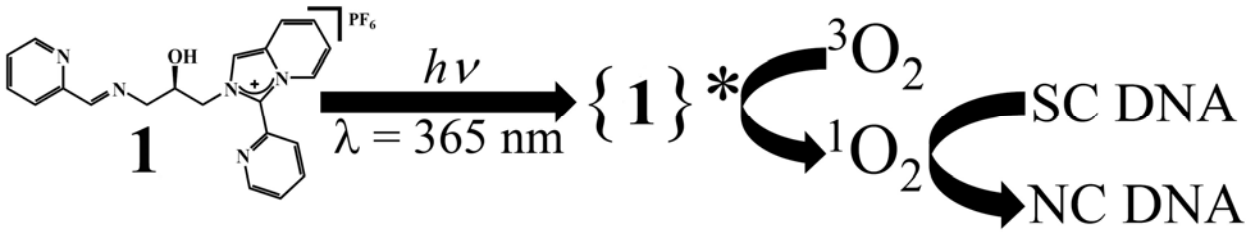
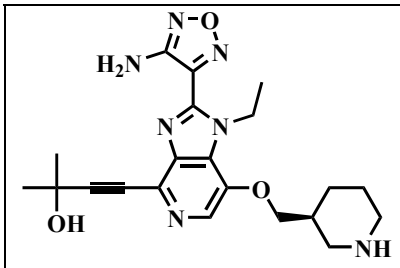


Fig. S16. Generation of ROS in treated HeLa cells as measured by DCF fluorescence. HeLa cells were treated with either **1** (4 μM)or in the presence of metal ions (50 μM) for 12 h in dark followed by photoirradiation with UV-A light of 365 nm (fluence rate: 610 μW cm^{-2} for 15 min) to provide a total dose of 0.55 *J* cm^{-2} . Cells were harvested using 5 mM sterile EDTA solution and washed once with ice-cold PBS by centrifugation at 60 × *g*, resuspended in the same and analyzed immediately by flow cytometry for green fluorescence at 530 nm. The mean fluorescence intensity (MFI) was plotted against treatment (mean ± s.d. (n=3). [H_2O_2] = 100 μM.

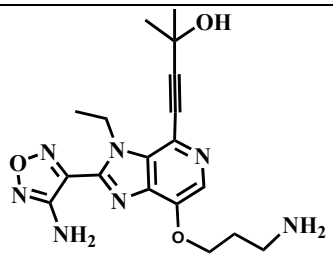


Scheme S1. Mechanistic scheme proposed for the DNA photo-cleavage activity of **1**.

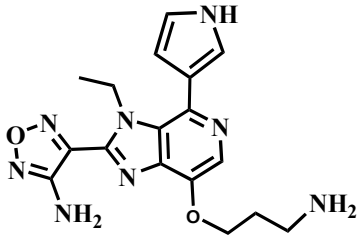
Chart S1. Literature reported imidazopyridine derivatives and their antitumor activity (reference numbers correspond to those given in the manuscript)



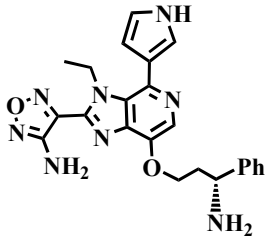
Cell line: breast carcinoma (BT474) IC_{50} = 9 nM; GSK3β inhibitor.[7]



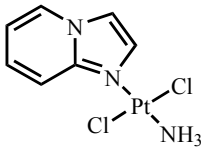
Cell Line: BT474 breast carcinoma IC_{50} = 1.05 μM; GSK3β inhibitor.[7]



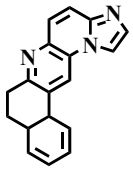
Cell Line: BT474 breast carcinoma IC_{50} = 2.76 μM; GSK3β inhibitor.[7]



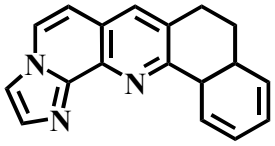
Cell Line: BT474 breast carcinoma IC_{50} = 0.1 μM; GSK3β inhibitor.[7]



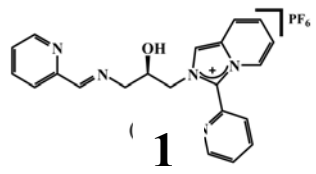
Cell line: A2780 (IC_{50} = 4.4 μM), A2780-cisR (IC_{50} = 2.34 μM)[8]



Cell Line: HT1080 (IC_{50} = 13.3 μM), HT29 (IC_{50} = 37.9 μM), M21 (IC_{50} = 38.1 μM), MCF7 (IC_{50} = 2.2 μM)[8]



Cell Line: HT1080 (IC_{50} = 13.4 μM), HT29 (IC_{50} = 7.2 μM), M21 (IC_{50} = 8.1 μM), MCF7 (IC_{50} = 6.6 μM)[8]



1

Cell lines: HeLa: (IC_{50} = 4.79 μM, IC_{50} = 0.71 μM in UV-A light); HepG2: (IC_{50} = 6.02 μM, IC_{50} = 2.07 μM in UV-A light) (This work)

Table S1. DNA binding parameters for **1**

Compound	Absorption titration	DNA melting	Isothermal Titration Calorimetry				
	K_b[a] / M^{-1} [s]	ΔT_m[b] / °C	N[c]	K[d] / M^{-1}	ΔH[e] / kJ mol^{-1}	ΔG[f] / kJ mol^{-1}	$T\Delta S$[g] / kJ mol^{-1}
1	5.7(±0.4) x 10^4 [0.26]	1.7	0.36	8.6 x 10^4	-13.9	-27.3	13.4

[a] Equilibrium DNA binding constant and *s* is the binding site size determined from the UV-visible absorption titration. [b] Change in DNA melting temperature. [c] The binding stoichiometry (*N*) determined from the ITC data. [d] Bindnig constant obtained from the ITC experiment. [e] The enthalpy change in DNA binding. [f] Change in Gibb's free energy. [g] ΔS, change in entropy.