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New Fe(III) and Co(II) Salen Complexes with Pendant Distamycins: Selective Targeting of Cancer Cells by DNA Damage and Mitochondrial Pathways

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Additional Synthetic Procedures.

Compound 5 (S-Fe). N,N'-Bis[4-[[3-(trimethylammonio)propyl]oxy]salicylidene]ethylene-diamine dibromide[1] (60 mg, 0.091 mmol) was taken in 5 mL of dry methanol to which $FeCl_3.6H_2O$ (28 mg, 0.103 mmol) was added and the resulting solution was heated at 60 °C for 12 h. The solvent was removed under vacuum and the resultant solid was dried (54 mg, 79%). IR (KBr): 3789, 3702, 2922, 2853, 2707, 2554, 2065, 1740, 1596, 1518, 1477, 1411, 1375, 1301, 1227, 1122, 1024, 952 cm^{-1}; HRMS: *m*/z = 577.2447 $[(M-2Br-Cl)+Na]^+$; Calcd. = 577.2444 $[(M-2Br-Cl)+Na]^+$. Anal. ($C_{28}H_{42}Br_2ClFeN_4O_4$) calcd: C 44.85, H 5.65, N 7.47; found: C 44.97, H 5.66, N 7.45.

Compound 6 (S-Co). The synthesis has been performed and all the characterization matched with the reported literature.[1]

Oligonucleotides. HPLC purified oligonucleotides (ODN) $[poly(dG-dC)]_2$, $[poly(dA-dT)]_2$, and CT DNA were purchased from Sigma, Genosys, Bangalore. The purity of the ODN was confirmed using high resolution sequencing gel. The concentrations of the *ds*-DNA were determined spectrophotometrically and expressed as base-pairs with ε values (M^{-1} cm^{-1}): $[poly(dA-dT)]_2$; ε_{262} = 13200, $[poly(dG-dC)]_2$; ε_{262} = 16800, and calf thymus (CT) DNA; ε_{258} = 13600. Stock solutions of the metal complexes of concentration ~2 mM in DMSO were diluted in appropriate buffer prior to use.

UV-Vis Absorption Spectral Titrations. To determine the extent of binding of the metal complexes toward *ds*-DNA, absorption titrations were carried out with CT DNA, $[poly(dA-dT)]_2$, and $[poly(dG-dC)]_2$. All titrations were performed in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4.

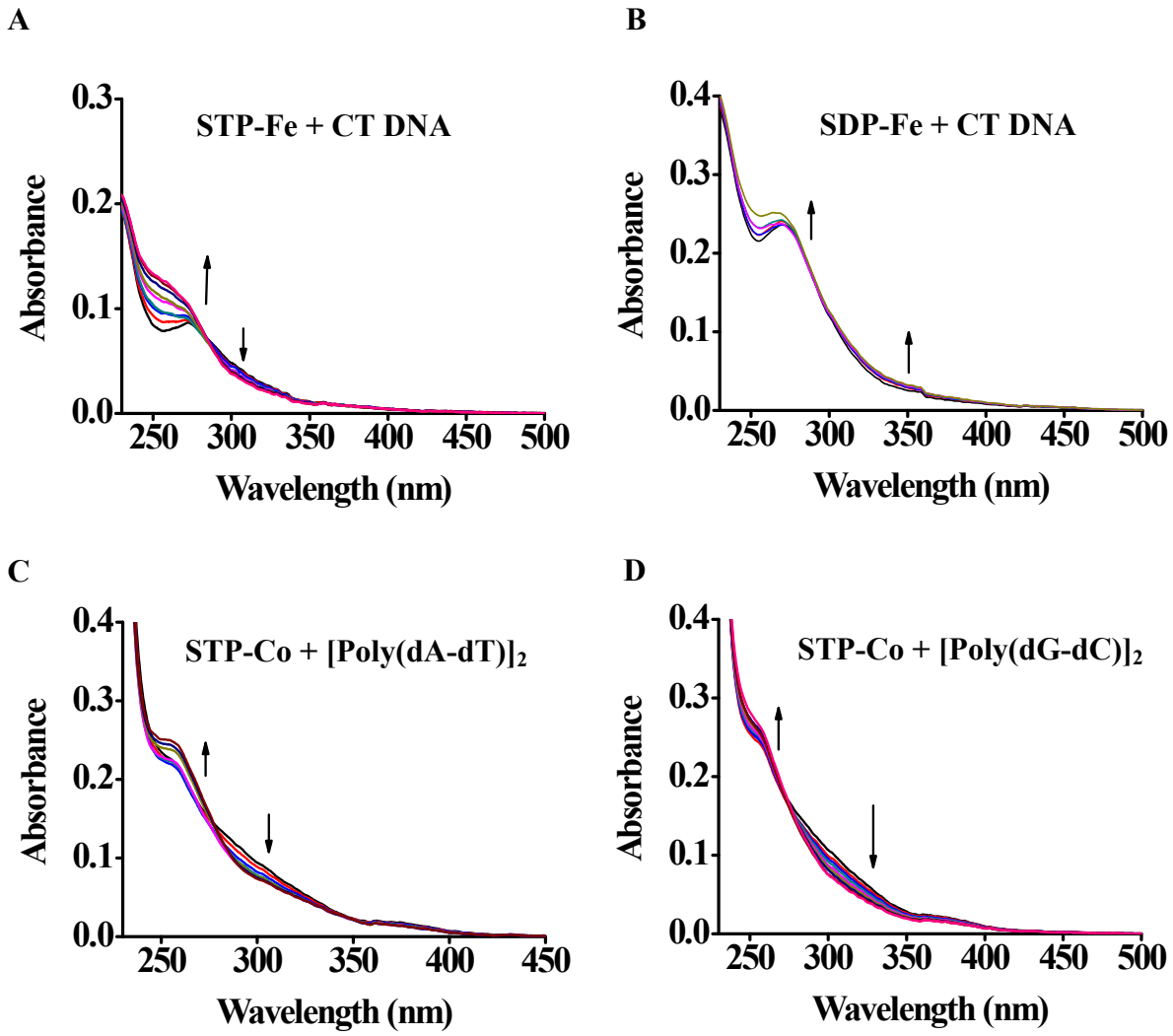

Figure S1. UV-Vis absorption titrations of (A) **STP-Fe** and (B) **SDP-Fe** with CT DNA and **STP-Co** with (C) [poly(dA-dT)]$_2$ and (D) [poly(dG-dC)]$_2$, respectively. All titrations were performed in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4.

Ethidium bromide and Hoechst Displacement Assays. To a solution of EtBr (1 μM), first increasing concentrations of CT DNA were added gradually to reach the saturation. The metal complex solution was then added progressively to this EtBr-DNA complex to displace EtBr from the *ds*-DNA. The sample was excited at λ_{ex} 526 nm and the decrease in the

fluorescence intensity was measured at 597 nm. The apparent binding constant (K_{app}) was determined as $K_a = K_{app}[L_{50}]$, where K_a is the binding constant of EtBr with CT DNA (2.6 $\times 10^5$ M^{-1})[2] and $[L_{50}]$ is the concentration of metal complex required for a reduction in the fluorescence intensity by 50%.

Similarly Hoechst displacement assay was carried out[3] where a Hoechst 33258 solution (1 μM) was taken and to it, CT DNA was added until saturation was reached. Each metal complex solution was then added to this Hoechst-DNA complex in order to replace Hoechst from the DNA minor groove. The reduction in the fluorescence intensity was determined at 460 nm with an excitation at λ_{ex} 355 nm. The apparent binding constant (K_{app}) was determined as $K_a = K_{app}[L_{50}]$, where K_a is the binding constant of Hoechst 33258 with CT DNA (9.6 $\times 10^5$ M^{-1})[4] and $[L_{50}]$ is the concentration of the metal complex required for quenching the fluorescence intensity by 50%. All the displacement assays were performed in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4 at 25 °C.

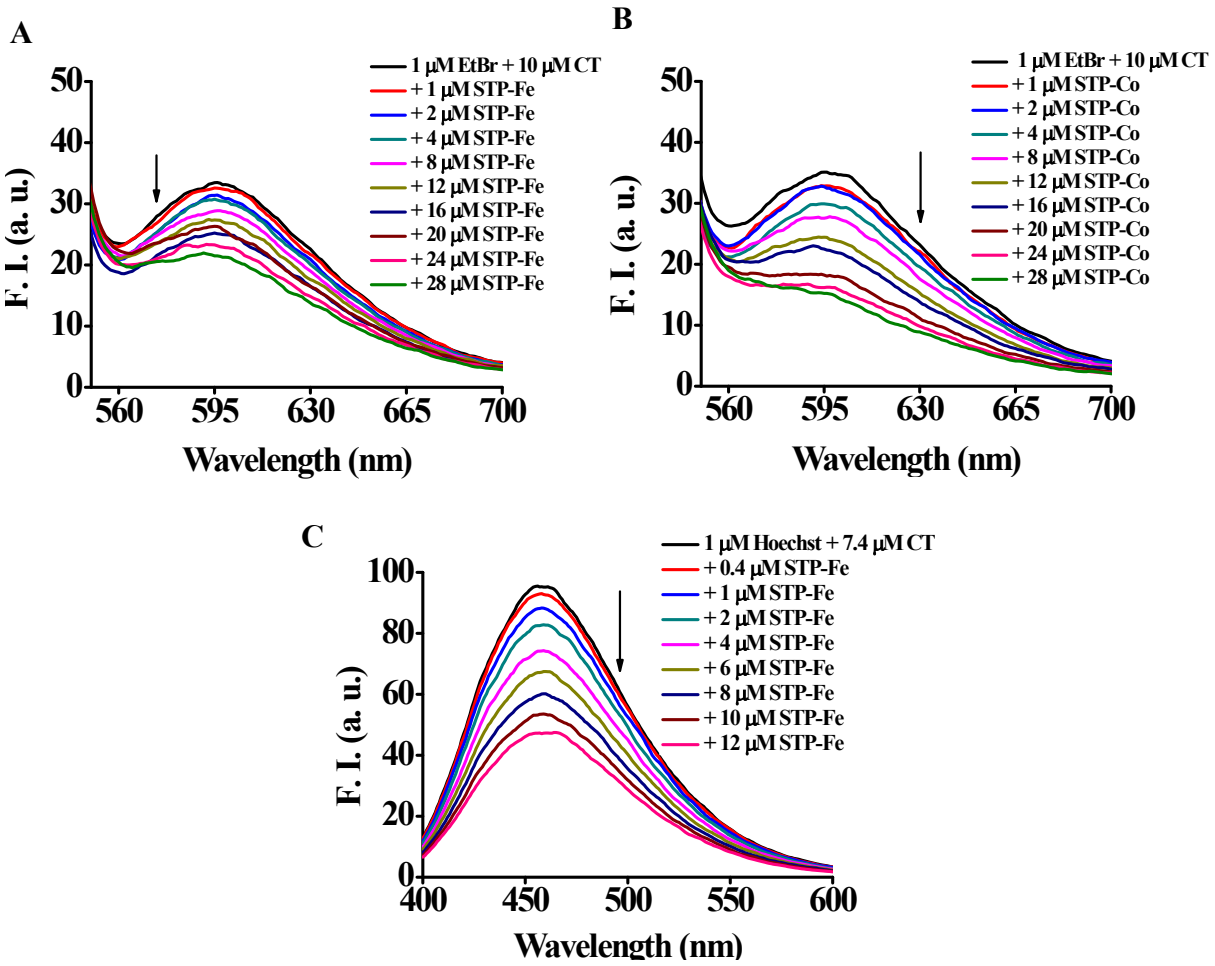

Figure S2. Ethidium bromide (EtBr) displacement assay with (A) **STP-Fe** and (B) **STP-Co**. Hoechst 33258 displacement assay with (C) **STP-Fe** .

Viscosity Measurements. For the viscosity measurement, we used a 200 μM CT DNA in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4 at 25 °C.[5] The metal complex solutions were added in increasing concentrations from higher stock solutions to ensure that the final volume did not increase above 5% of the initial volume. An equilibration time of 20 min was observed before the measurement of the readings. The reported data are the average of several readings within ± 0.04 s. The viscosity of the free DNA and complex bound DNA solutions has been related to the length of the DNA helix according to equation 1.

$L/L_0 = [\eta]^{1/3}/[\eta_0]^{1/3} = (t\text{-}t_b)^{1/3}/(t_0\text{-}t_b)^{1/3}$.....1

where L is the length of complex bound DNA, L_0 is the length of free DNA, η is the viscosity of complex bound DNA, η_0 is the viscosity of free DNA, t is the flow time of the solution containing complex bound DNA, t_0 is the flow time of the solution containing free DNA and t_b is the flow time of buffer.

Circular Dichroism (CD) Spectroscopy. The CD spectra were recorded on a JASCO J-810 CD spectropolarimeter with a Peltier temperature controller at a scanning speed of 50 nm/min with a quartz cell of 10 mm path-length. To a 40 μM CT DNA, aliquots of the metal complex solutions prepared in 10 mM Tris-HCl, 40 mM NaCl, pH 7.4 buffer were added with an equilibration time of 15 mins and the experiment was pursued until saturation was obtained.

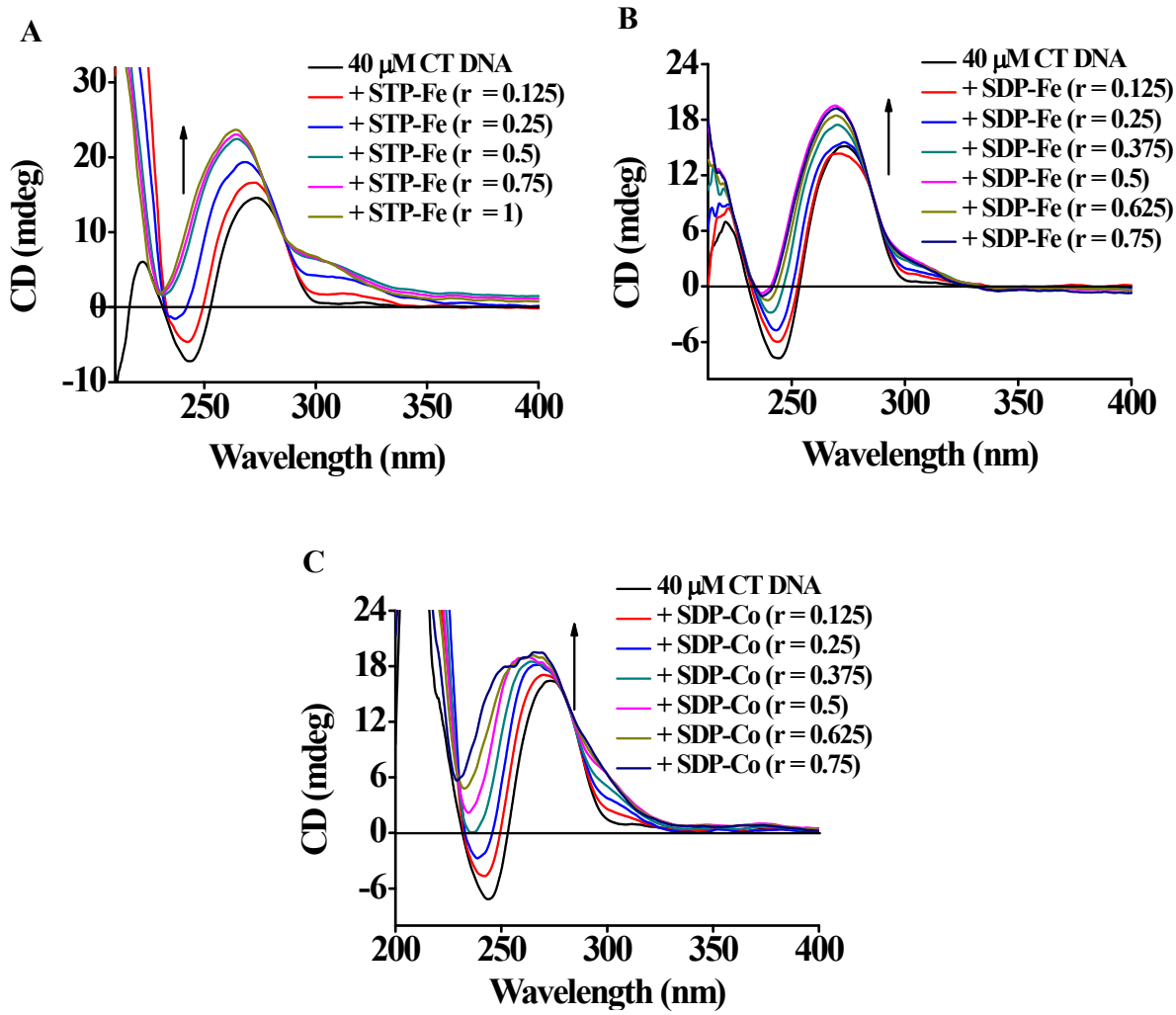

Figure S3. CD spectral titrations of 40 μM CT DNA with increasing concentrations of (A) **STP-Fe** (B) **SDP-Fe** and (C) **SDP-Co** in a buffer having 10 mM Tris-HCl, 40 mM NaCl, pH 7.4.

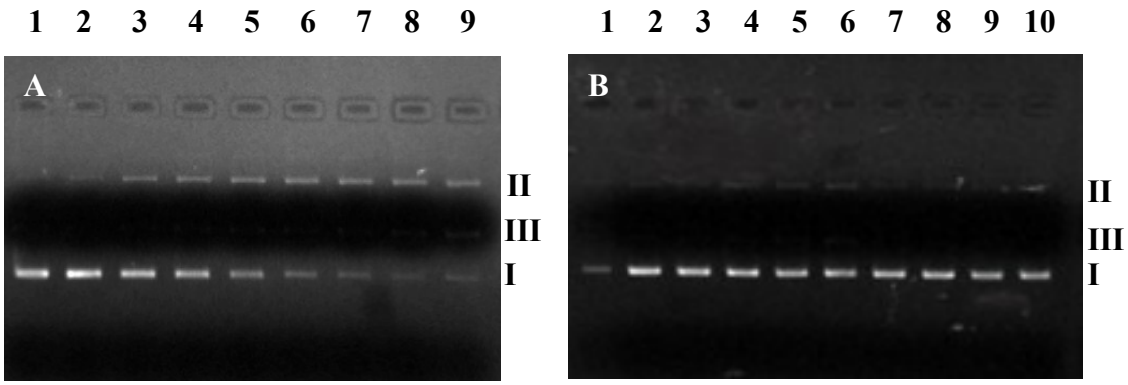

Figure S4. Agarose gel electrophoresis showing metal complex mediated cleavage of plasmid DNA, pUC18. (A) lane 1: DNA alone; lane 2: DNA with DTT (2 mM); lanes 3-9: DNA with DTT and increasing concentrations of **STP-Co** (1, 2, 5, 7, 10, 15, and 20 μM) and (B) lane 1: DNA alone; lane 2: DNA with DTT (2 mM); lanes 3-10: DNA with DTT and increasing concentrations of **STP-Fe** (0.5, 0.75, 1, 2, 3, 4, 5, and 10 μM). Supercoiled plasmid DNA pUC18 (0.1 μg/reaction) was incubated with varying metal complex concentrations at 37 °C for 30 min. Forms I, II, and III represent supercoiled, nicked circular, and linear form, respectively of the plasmid DNA.

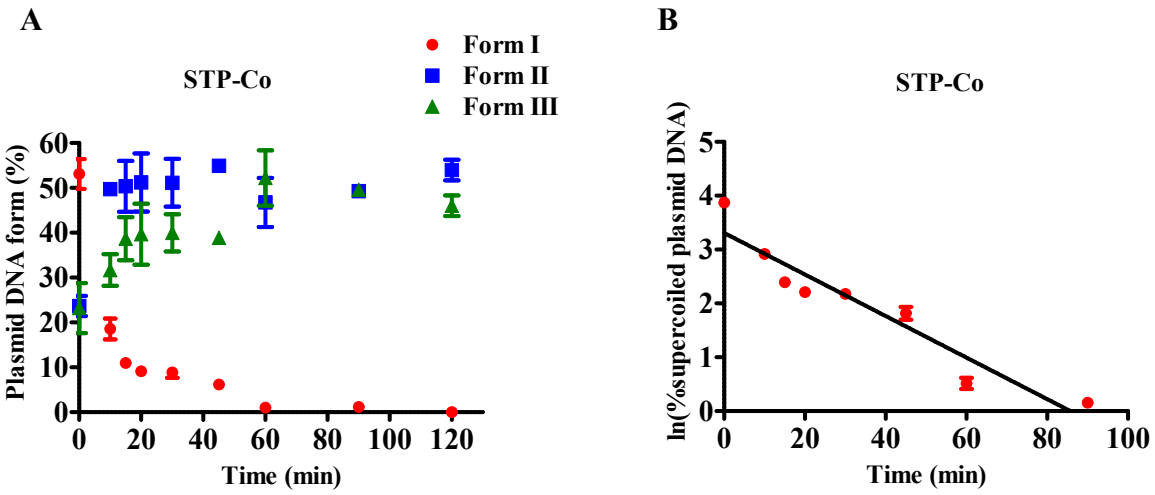

Figure S5. Plasmid DNA cleavage by **STP-Co** as a function of the reaction time. (A) Variation of the relative concentrations of forms I, II, and III. Forms I, II, and III represent supercoiled, nicked circular, and linear forms, respectively of the plasmid DNA. (B) Pseudo-first order kinetics of plasmid DNA cleavage.

Cell Culture. HeLa (human cervical cancer cells), HEK 293T (human embryonic kidney transformed cells), A549 (adenocarcinoma human alveolar basal epithelial cells), MCF7 (human breast cancer cells), MCF10A (normal mammary epithelial cells) and HFF (human foreskin fibroblast cells) were grown in Dulbecco's modified Eagles media (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma), 2 mM L-glutamine and Penicillin/Streptomycin (100 units and 0.1 mg/mL) in a humidified CO_2 incubator maintained at 5% CO_2. Other than the primary cell line, all the other cells were passaged 3-4 times a week using Trypsin-EDTA.

Cell Viability Assay..HeLa (human cervical cancer cells), HEK 293T (human embryonic kidney transformed cells), A549 (adenocarcinoma human alveolar basal epithelial cells) and HFF (human foreskin fibroblast) cells were seeded in 96-well plates (5.0×10^3/well) and allowed to adhere over a period of 24 h prior to their exposure to different concentrations of the various salen complexes in presence of 0.2% FBS containing DMEM. After an incubation of 72 h, 25 μL of a 4 mg/mL solution of methylthiazolyltetrazolium bromide (MTT) reagent was added to each well and incubated with the cells for an additional 4 h. Following this, the supernatants were discarded leaving behind Formazan crystals in each well which were dissolved in DMSO, and absorbance read at a λ_{max} of 570 nm. A similar MTT assay was carried out for MCF7 and MCF10A cells with a seeding density of 10,000 cells per well and an incubation time of 96 h of the cells with the complex.[6] The %cell viability was calculated from the absorbance values. The percent cell viability, as a function of test compound concentration was plotted and IC_{50} of the compounds were elucidated. The concentration of the compounds at which the conversion of MTT to formazan by viable cells is reduced to 50% in comparison to control cells is defined as the IC_{50}. Each experiment was performed in three parallel replicates and experiments were repeated at least three times (n = 3).

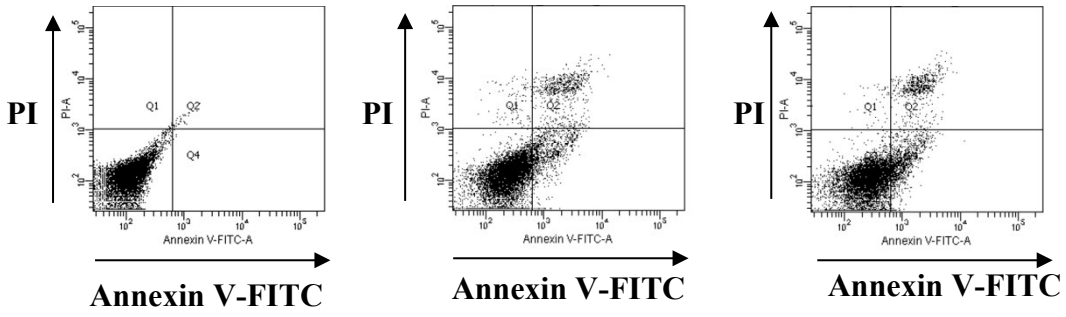

Figure S6 Representative dot plots for Annexin V and PI staining of (A) untreated (HEK 293T) and after incubation with (B) **S-Fe** and (C) **S-Co** for 12 h at near IC_{50} concentration. The stages of the cells were assigned as viable (LL), early apoptotic (LR), late apoptotic (UR), and necrotic cells (UL) in the four quadrants.

Fluorescence and Confocal Microscopy. For DAPI staining, MCF7 cells were plated on coverslips, placed in 6-well plates, at a seeding density of ~10,000 cells per well. After overnight incubation in a humidified CO_2 incubator maintained at 5% CO_2 at 37 °C, the cells were treated with the different metal complexes at a concentration nearly equal to the IC_{50}. After 16 h of incubation with the appropriate complexes, the cells were fixed using 4% paraformaldehyde, treated appropriately and mounted on the glass slides using vectashield mounting medium with DAPI (vector labs). The mounted coverslips on the glass slides were sealed *via* nailpaint and then viewed under fluorescence Microscope using 100 X oil immersion objectives.

To observe the effect of the metal complexes on the cellular morphology, HEK 293T and A549 cells were plated at a density of 5 X 10^4 cells/well in a Nunc™ 24-well plate one

day prior to treatment. After 48 h of treatment with the range of concentrations of the various metal complexes, the cells were imaged under 20X phase contrast objective.

In order to visualize the nuclear fragmentation, HEK 293T and A549 cells were plated on 18 mm coverslips, placed in a Nunc™ 12-well plate, at a seeding density of 15,000 cells per well. After 24 hours, these were treated with the different metal complexes at a concentration nearly equal to the IC_{50}. After 24 h of incubation with the complexes, the cells were fixed using 4% paraformaldehyde, washed with 50 mM PBS and the cover slips were mounted on the glass slides using Prolong® Gold Antifade reagent with DAPI. These were sealed on to the glass slide using nail paint and then viewed under 63X oil immersion objective. Z-stack over 4 μm thickness was taken to cover the entire cell volume.

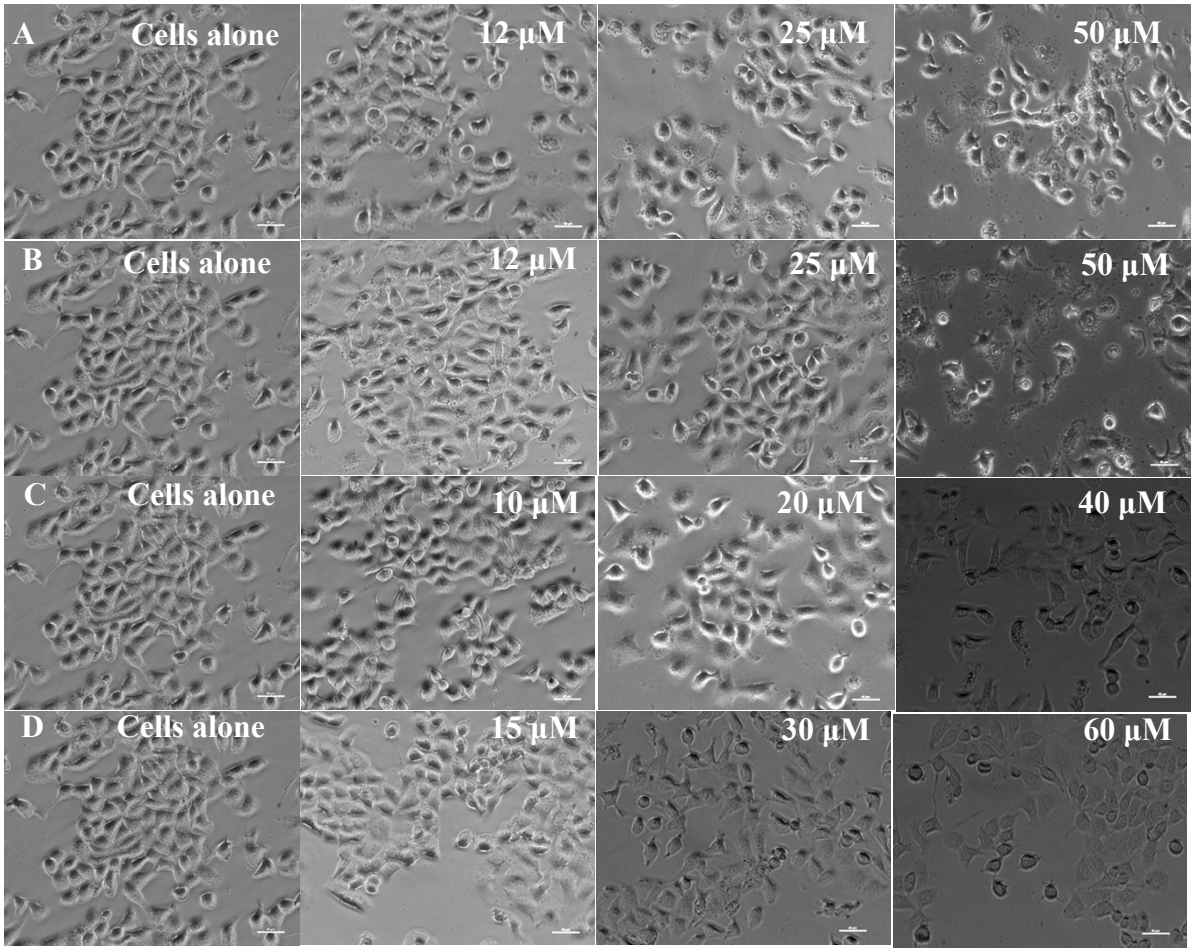

Figure S7. Representative bright field images of cell morphology on treatment with complexes (A) **SDP-Co** (B) **STP-Co** (C) **SDP-Fe** and (D) **STP-Fe** at different concentrations for 48 h. Panels A, B, C, and D (left to right) represent untreated cancer A549 cells and treatment with the metal complexes at increasing concentrations, respectively.

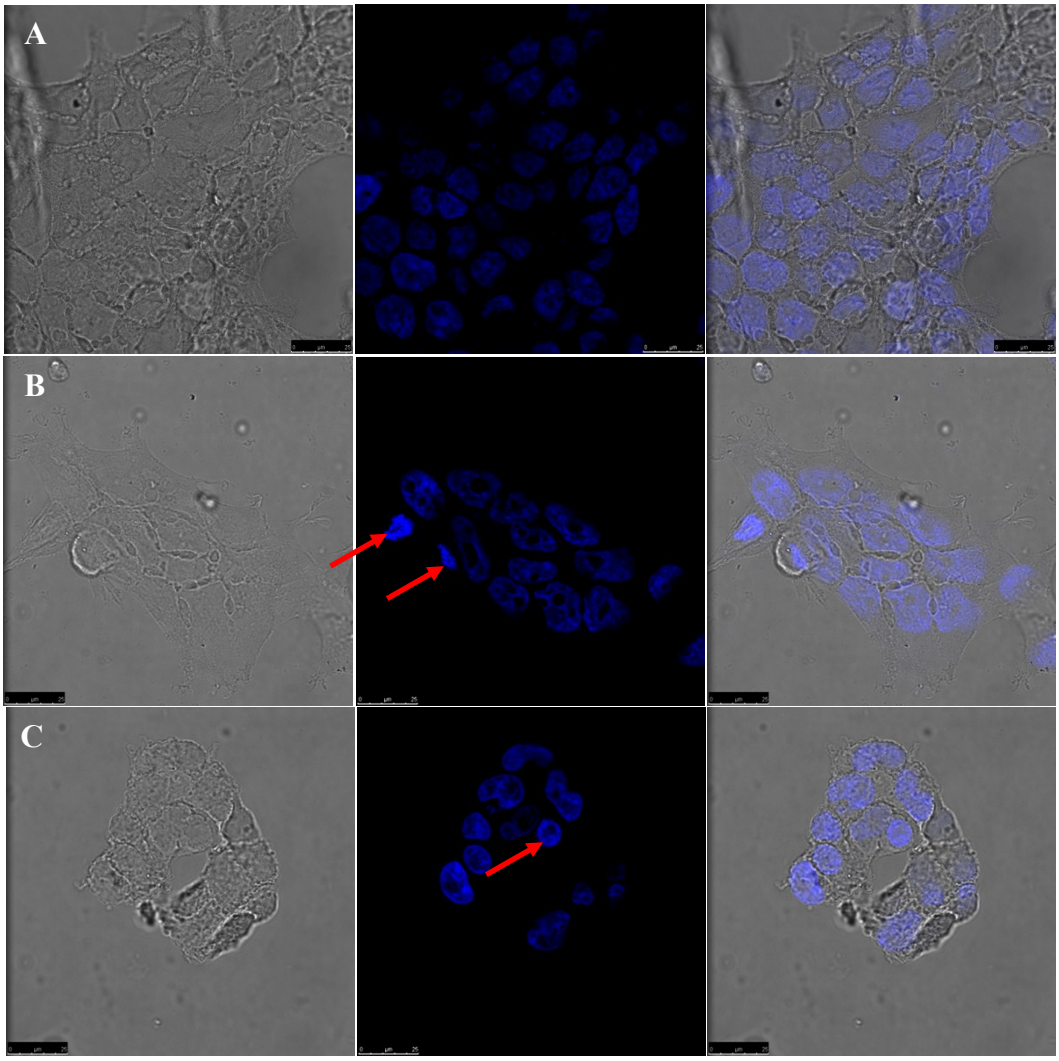

Figure S8. Representative confocal microscopic images of (A) untreated HEK 293T cells and on treatment with (B) **SDP-Fe** and (C) **STP-Fe** at a concentration nearly equal to the IC_{50} for 24 h. DAPI was used as a nuclear counterstain. Panels A, B, and C (left to right) represent bright field, DAPI nuclear counterstain (blue) and overlay of the previous two images, respectively. Scale bar = 25 μm.

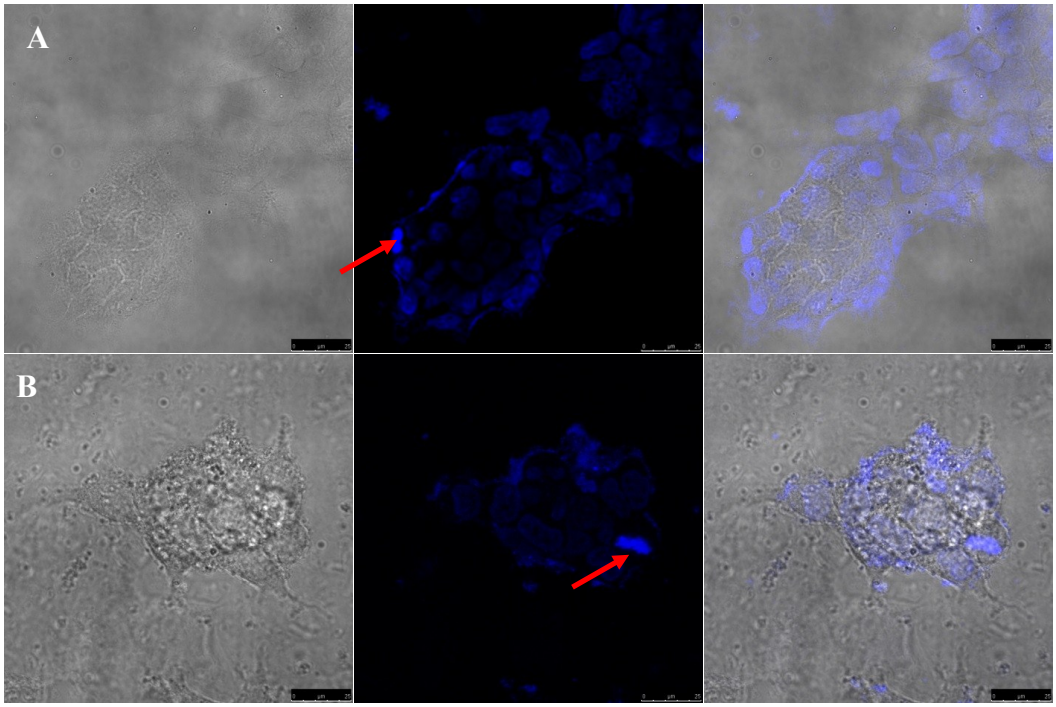

Figure S9. Representative confocal microscopic images of HEK 293T cells on treatment with (A) S-Fe and (B) S-Co at a concentration nearly equal to the IC_{50} for 24 h. DAPI was used as a nuclear counterstain. Panels (left to right) represent bright field, DAPI nuclear counterstain (blue) and overlay of the previous two images, respectively. Scale bar = 25 µm.

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