Mitochondria Topoisomerase 1 targeted anticancer therapy using Irinotecan encapsulated mesoporous MIL-101(Fe) synthesized via vapour assisted method

Anirban Ghosh[ǂ,1], Arijit Ghosh[ǂ,2], Arpan Bhattacharyya[2], Riddhi Mitra[1], Benu Brata Das[*,2] and Asim Bhaumik[*,1]

[ǂ]These authors contributed equally.

MATERIALS AND METHODS

Sample preparation for morphological analysis:

It was found that both the MIL-101(Fe) and MIL-Iri are well dispersed in aqueous solution. The aqueous soup of MIL- 101(Fe) and MIL-Iri is drop casted over a carbon coated TEM grid and a previously cleaned silicon wafer. The dried grid was tested in FEG-TEM analysis and the dried silicon wafer was performed FE-SEM analysis.

Cell culture, drugs, reagents and antibodies

Cells were cultured at 37°C with 5% CO_2 in DMEM containing 10 % FBS (Life Technologies). Human cancerous cell line MCF7 and HCT116 were obtained from the Developmental Therapeutics Program as a kind gift from Dr. Yves Pommier (NIH/NCI/USA). Irinotecan (I1406) was purchased from Sigma Aldrich (USA). Anti-mitochondrial Topoisomerase1 specific antibody[1] (Top1mt, polyclonal mouse) was a kind gift from Dr. Yves Pommier (Centre for Cancer Research, NCI, NIH, USA). Mouse monoclonal antibodies: dsDNA (Ab27156) was purchased from Abcam (Cambridge, USA). Antihuman Top1 (C21) and secondary antibodies: HRP-conjugated anti-rabbit IgG or anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). MitoTracker green (M7514) was obtained from Molecular probes.

Live cell confocal microscopy

Live cell imaging was carried out as described previously.[2] Briefly indicated cells were grown on confocal dishes (Genetix, Biotech Asia Pvt. Ltd.). Fluorophores were excited using either

separately or in combination with UV (Diode 405), 488 nm (Argon), 561 nm (DPSS561) or 633 nm (HeNe633) laser lines using confocal laser-scanning microscope (Leica TCS SP8) with 63X/1.4 NA oil objective equipped with a heated environmental chamber set to 37°C with an optimal CO_2 facility. The percentage of cells displaying the indicated fluorescence was determined with Adobe Photoshop 7.0 from at least 20-25 cells expressing individual constructs.

Preparation of mitochondria

Mitochondria were prepared as described previously.[3] Briefly, cell pellets were suspended in 10 mM NaCl, 1.5 mM $CaCl_2$, and 10 mM Tris-HCl (pH 7.5) at 25°C for 5 min. Following osmotic shock, cells were homogenized using a glass Dounce homogenizer and mixed with stabilizing buffer [2 M sucrose, 35 mM EDTA, and 50 mM Tris-HCl (pH 7.5) at 25°C]. Cell lysates were centrifuged at 750 × g for 5 min to separate the nuclear fraction and cell debris. Mitochondria were spun down from the supernatant at 10,000 × g for 20 min, washed three times with MT buffer [250 mM sucrose, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and 20 mM Hepes-KOH (pH 7.4) at 25°C], and re-suspended in MT buffer.

Immunocomplex of enzyme (ICE) bioassay

Detection of Top1mt and nuclear Top1-cleavage complexes by ICE Bioassay was performed as described previously.[1] Briefly cells (5 x 10^6) either untreated or treated with the indicated concentration of drugs were subjected to mitochondria isolation as described previously where cells were lysed by adding lysis buffer (6 M Guanidinium Thiocyanate, 10 mM Tris-HCL, pH 6.5, 20 mM EDTA, 4% Triton ×100, 1% sarcosyl, and 1% DTT). Mitochondrial lysates were mixed with 0.4 ml of 100% ethanol, incubated at –20°C for 5 min, and cleared by centrifugation (12,000 g; 10 min). Supernatants were discarded and pellets washed two times with 100%

ethanol and then dissolved in 0.2 ml of 8 mM NaOH (freshly made) and sonicated for 10–20 s at 20% power. For immunodetection, mtDNA at varying concentrations were spotted onto a nitrocellulose membrane (Millipore, USA) using a slot-blot vacuum system (Biorad, USA). For detection of nuclear Top1cc, whole cells were lysed by adding lysis buffer, cleared by centrifugation and the pellet was dissolved in 8 mM NaOH and slot blotted as indicated above. Immunoblotting was carried out with anti-Top1mt or anti-nuclear Top1 specific antibodies. Anti-dsDNA was used for loading control. Immunoblots were visualized using ECL chemiluminescence reactions on a ChemiDoc™ MP System.

Cell survival assays

Cell survival was carried out as described previously.[4] Briefly, MCF7 (human breast adenocarcinoma; 1×10^3) and HCT116 (human colorectal carcinoma; 1×10^3) cells were seeded in 96-well plates (BD Biosciences, USA). After 24 h, cells were treated with the indicated drugs and kept for a further 48 h. Cell survival was then assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, M5655). Plates were analyzed on a Molecular Devices Spectra Max M2 Microplate Reader at 570 nm. The percent inhibition of viability for each concentration of drugs was calculated with respect to the control. Data represent the mean values ± S.D. for three independent experiments.

Quantification of mtDNA Damage

Long range PCR is a method to amplify the DNA fragment of more than 5Kb using a special kind of high-fidelity DNA polymerase. To compare the levels of mtDNA damage and repair in MCF7 cells treated with or without MIL-Iri for the indicated times, or further cultured in drug-free medium to measure repair using mtDNA long-range polymerase chain reaction (PCR) as

described previously.[2,3] We used human mtDNA sequence specific primers to amplify a 9 kbp fragment of mtDNA respectively. A small 110 base pair for human specific mtDNA fragment was also amplified for normalization. PCR reactions were limited to 18 cycles, to ensure that the amplification process was still in the exponential phase. The damage index is determined by the ratio LR/SR of long-range PCR product (LR) by the short-range PCR product (SR). The sequences of the primers are listed in Table below.

Gene Name	Species	5'-Forward Primer	5'-Reverse Primer
LRMT	Human	TTTCATCATGCGGAGATGTTGGATGG	TCTAAGCCTCCTTATTCGAGCCGA
SRMT	Human	CCCACAAACCCCATTACTAAACCCAC	TTTCATCATGCGGAGATGTTGGATGG

Determination of intracellular uptake of MIL-101(Fe) or MIL-Iri using flow cytometry

Cells were incubated with MIL-101(Fe) or MIL-Iri for indicated time at 37°C.[5] Then cells were trypsinized and washed or resuspended in PBS. The intracellular fluorescence (blue or red emission) was analysed by flow cytometry (BD, FACSARIA III).

Cell apoptosis and necrosis analysis

The MCF7 cells were cultured in 60 mm dishes either treated or untreated for indicated time periods were trypsinised, washed with PBS and centrifuged at 3000 rpm for 5 min. Then, cells were resuspended in 500 µL binding buffer and stained with Annexin V-FITC and PI according to the protocol of BD Biosciences. The cells were incubated in the dark at room temperature for 15-30 min. Finally, the percentage distribution of apoptotic and necrotic cells was analysed by flow cytometry (BD, FACSARIA III).[6]

Cellular Fe content estimation by ICP-MS

Total cellular Iron (Fe) content was measured by ICP-MS method. Healthy MCF7 cells were grown in 5% FBS supplemented media (DMEM) and were seeded in six well plates and treated with MIL-101(Fe) or 300nM of MIL-Iri for 3 hrs. After treatment, these cells were trypsinised and counted in Hemocytometer. Briefly $1x10^6$ cells were fixed in 70% ethanol, resuspended in PBS and subjected to ICP-MS[7].

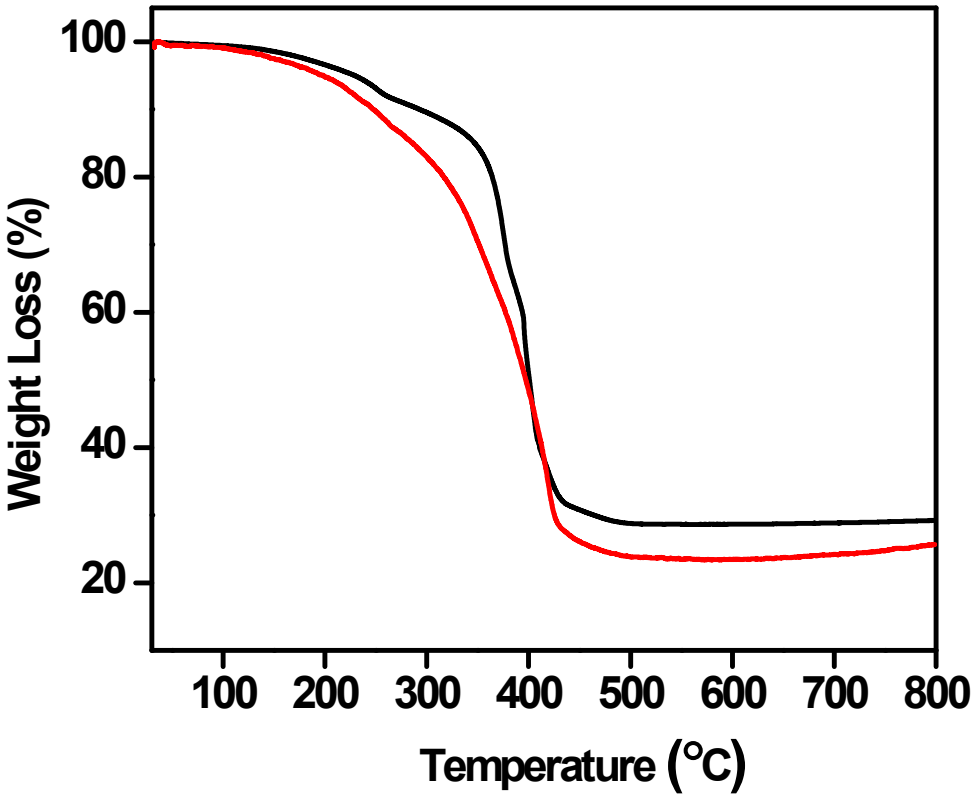

Figure S1: TGA analysis of MIL-101(Fe) (Black) and MIL-Iri (Red).

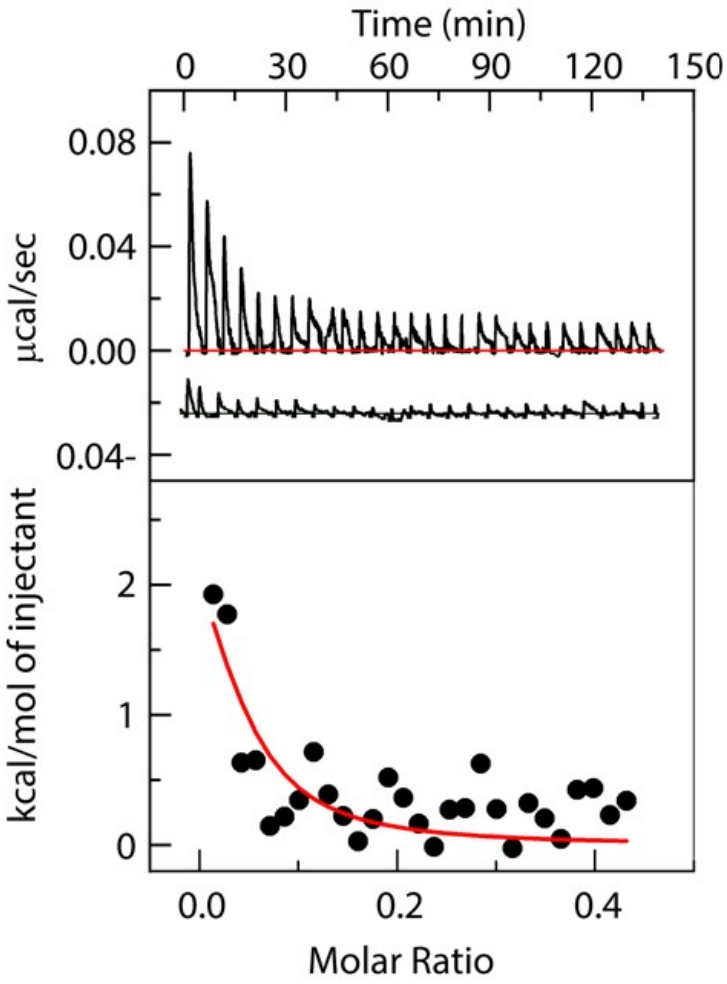

Figure S2: Calorimetric titration of Irinotecan with MIL-101(Fe), Upper Plot: Thermogram (thermal power required to maintain a zero temperature difference between reference and sample cells in the calorimeter), Lower Plot: Binding Isotherm

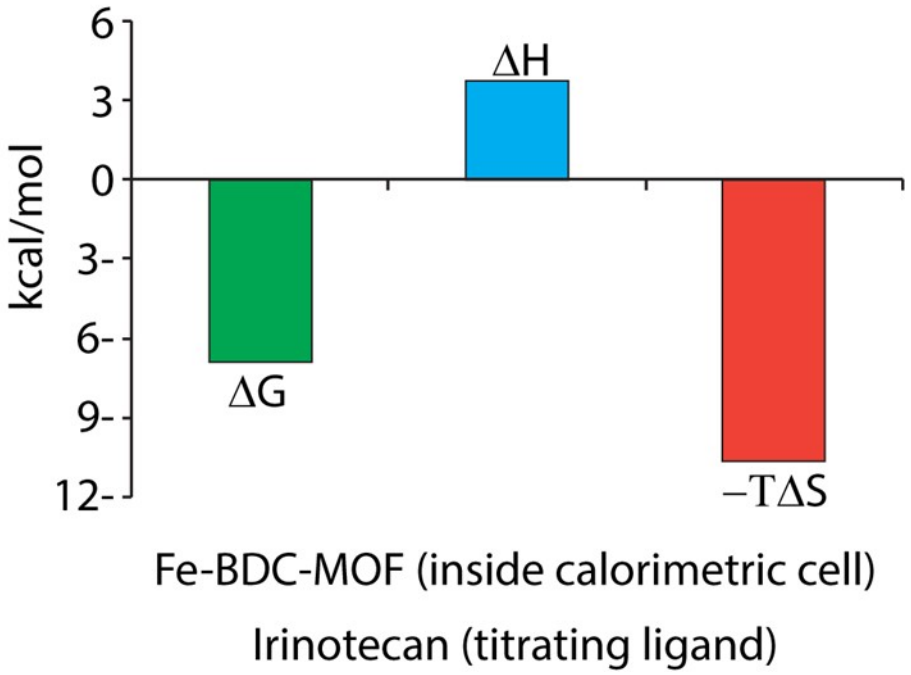

Figure S3: Thermodynamic binding profile for the Irinotecan-MOF interaction

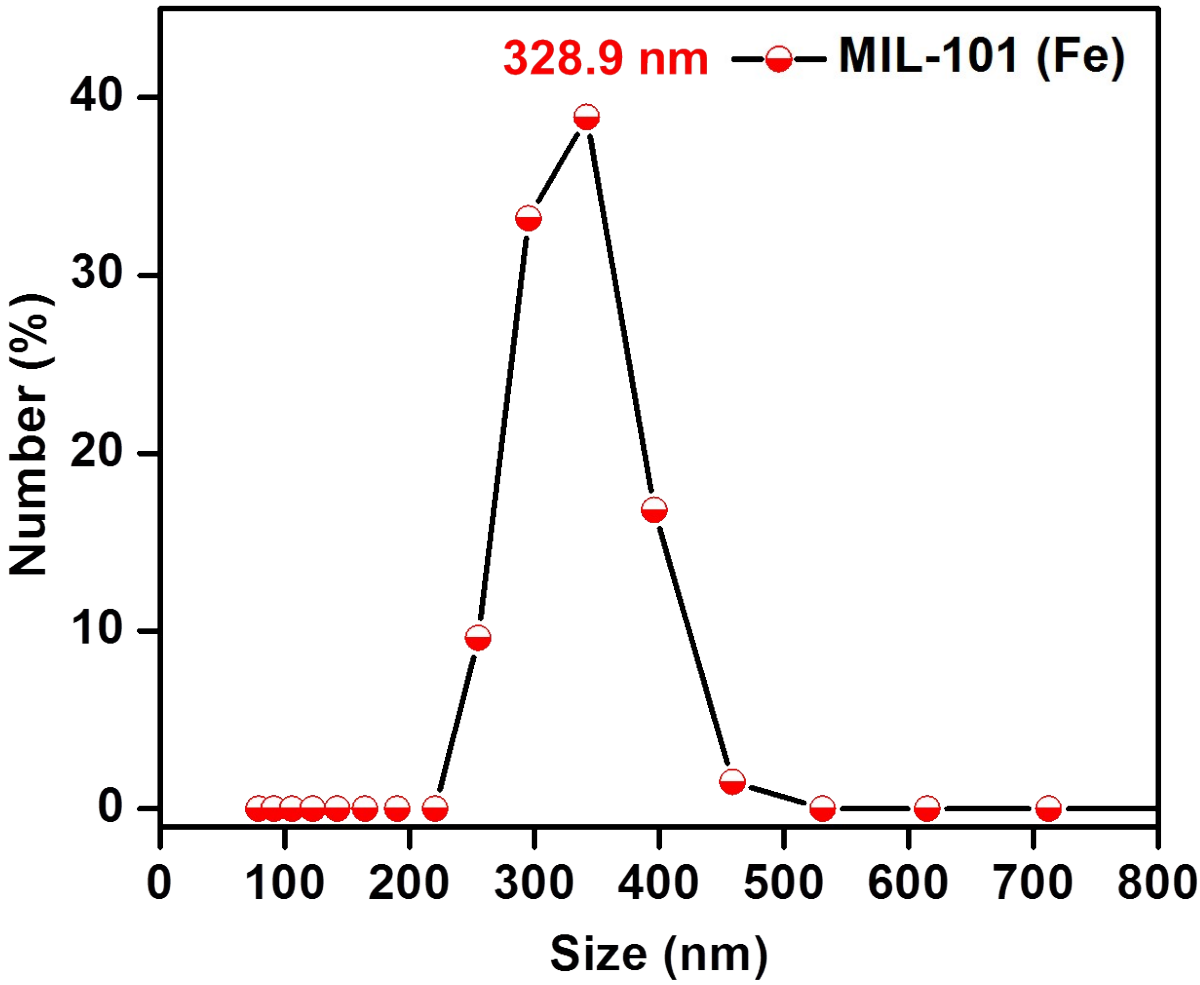

Figure S4: Hydrodynamic particle size MIL-101(Fe)

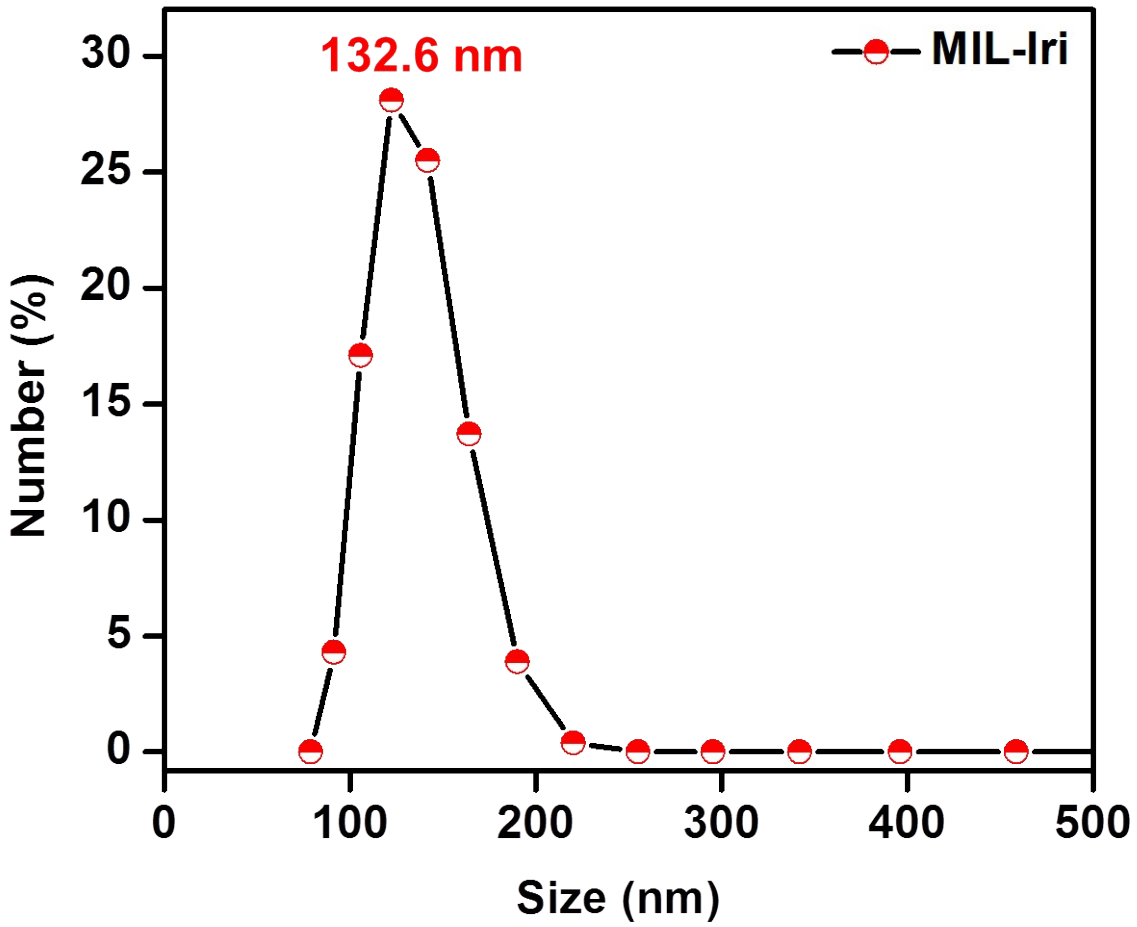

Figure S5: Hydrodynamic particle size MIL-Iri

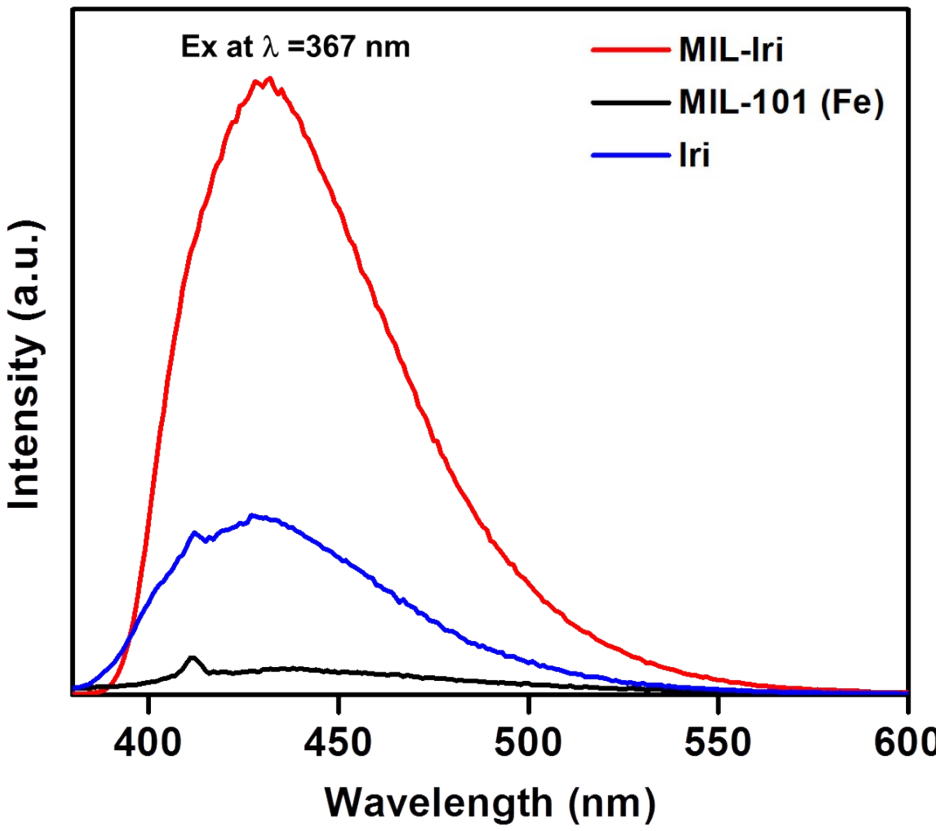

Figure S6: Photoluminescence (PL) Spectra of MIL-101(Fe) (Black), MIL-Iri (Red), and Irinotecan (Blue)

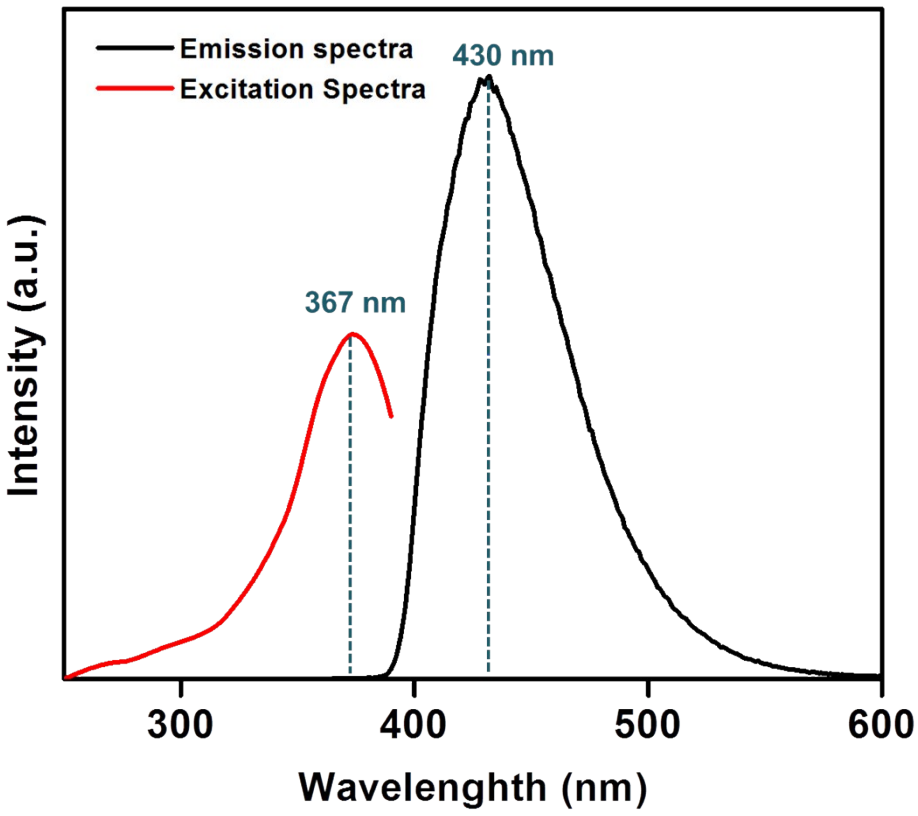

Figure S7: Convergence of excitation and emission spectra for MIL-Iri

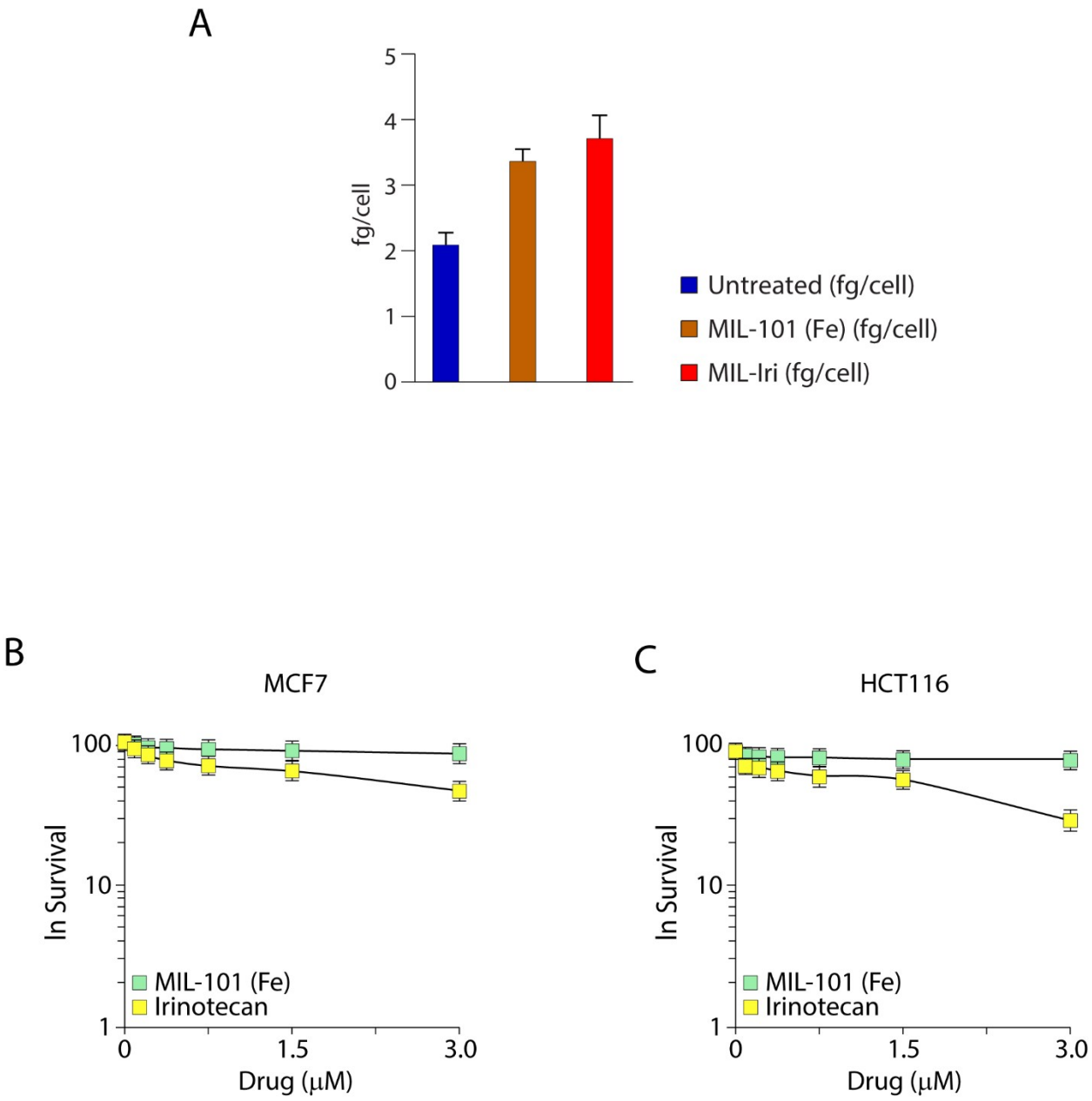

Figure S7: (A) Measurement of cellular Iron (Fe) content by ICP-MS. MCF7 cells either untreated or treated with MIL-101(Fe) or 300nM MIL-Iri for 3 hrs were subjected to ICP-MS. Error bars represent SDs (n = 3). **(B and C)** Cell survival curves of MCF7 (Breast cancer) and HCT116 (colon cancer) cells treated with Irinotecan. Drug induced cytotoxicity (%) was calculated with respect to the untreated control. Error bars represent SDs (n = 3).

References:

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