Electronic Supplementary Information (ESI)

Implantable HDAC-inhibiting Chemotherapeutics Derived from Hydrophobic Amino Acids for Localized Anticancer Therapy

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Materials and methods:

1-Naphthylacetic acid (1-NAA) was purchased from Spectrochem Chemicals, 1,1′-carbonyl diimidazole (CDI) and hydroxylamine hydrochloride were purchased from Alfa Aesar, Potassium hydroxide, hydrochloric acid, nitric acid, phosphoric acid, sodium nitrate, sodium dihydrogen phosphate, disodium hydrogen phosphate and triethylamine were obtained from Merck. Acetic acid and sodium acetate were purchased from SD Fine. Sulfuric acid was purchased from Himedia. Anhydrous sodium sulphate was obtained from Rankem. All solvents used in the synthesis were purified, dried, or distilled, as required. Vorinostat (Suberanilohyroxamic acid (SAHA)) was synthesized by reported protocol.[1] NMR spectra were recorded on Bruker Ultra Shield (400 MHz and 500 MHz) spectrometer. Mass spectra were recorded in HR-MS mode on MicroTOF-Q-II instrument manufactured by Bruker Daltonics; IR spectra were recorded by using PerkinElmer Spectrum BX FT-IR spectrometer. UV-vis absorption spectra were recorded on PerkinElmer Lambda 25. Scanning Electron microscopy (SEM) was performed on Ultra55 plus HR-SEM by Carl Zeiss. Circular dichroism (CD) was recorded on JASCO J-815 CD spectrometer. Fluorescence images were obtained on a Multiphoton Confocal system from Carl Zeiss and OLYMPUS CellSens Dimension Microscopy. Chemiluminescence detection was carried out with the ECL plus Western Blotting Detection System (Thermo Pierce). Cell cycle study and Annexin V-PI studied by flow-cytometry were used (BD FACS ARIA III). MTT assay was performed by microplate reader (Synergy-HT, Bio-Tek Instruments, Inc.).

General Synthetic procedures:

Methyl ester amino acid (1 eq.) was taken in dry THF (15 mL), and triethylamine (1.5 eq.) was added dropwise to it at 0 ˚C followed by stirring for 10 min. In a separate flask, 1-naphthalene acetic acid or phenyl acetic acid (1.1 eq.) and CDI (1.5 eq.) were taken in dry THF (15 mL) and stirred for 10-15 min. This activated acid solution was added dropwise at 0 ºC to the basic mixture of methyl ester of amino acid followed by stirring under N_2 atmosphere at RT for 6 h. The reaction was monitored by TLC (eluent phase was 5:95 v/v, MeOH:$CHCl_3$). THF solvent was removed from the reaction mixture under vacuum, and appropriate volume of $CHCl_3$ was added to it. The $CHCl_3$ layer was washed with dil. HCl and sat. $NaHCO_3$, dried over anhydrous Na_2SO_4. The organic solvent $CHCl_3$ was removed by rotary evaporator yielded crude product that was purified by flash chromatography (stationary phase was silica 100-200 mesh, and eluent phase was $CHCl_3$).

Synthesis of N-Acetyl-phenylalanine-methyl ester (AcF-OMe)[1]: To a stirred suspension of L-phenylalanine methyl ester hydrochloride (1 g, 4.65 mM, 1 eq.) methanol (10 mL) at 0 °C was added triethylamine (14 mM, 1.9 mL, 3 eq.), dropwise. The solution was stirred at room temperature for 30 minutes and then acetyl chloride (1.2 eq.) was added dropwise and the solution was stirred for 3 h at room temperature. The solvent was removed under reduced pressure by rotary evaporator, ethyl acetate (20 mL) was added and the mixture was purified through silica gel (100-20 mesh, 5% CH_3OH:$CHCl_3$). Removal of the solvent under reduced pressure afforded a colourless solid.

Synthesis of 2a: AcF-OMe (1 g) was dissolved in methanol (10 mL) followed by dropwise addition of solution of hydroxylamine (3 eq.) in potassium hydroxide (5 eq., pH of the reaction medium *ca*. 9) and methanol (5 mL) was solvent at 0 °C. Conversion was monitored by TLC ($CHCl_3$: CH_3OH = 94:06 v/v). After stirring for 12 h at RT, methanol was removed by rotary evaporator and the solid residue washed with ethyl acetate three times with constant stirring to remove the unreacted starting material. The precipitate was further washed with diethyl ether (10 ml x 3) and then decanted. A light-yellow colored solid was obtained at this stage, which was again washed with cold water to remove the excess salt and ethyl acetate, centrifuged and dried overnight under vacuum desiccator to obtain **2a** in 74% yield. ^{1}H NMR (400 MHz, DMSO-d6): δ 1.94 (3H, s), 2.89-2.93 (2H, 2.91 (d), 2.91 (d)), 4.87 (1H, t), 7.19 (1H, m), 7.24-7.34 (Ar-H); ^{13}C (100 MHz, DMSO-d6) δ (ppm): 169.68, 22.76, 38.37, 167.26, 129.1, 129.11, 128.92, 137.11, 53.61, 128.71, 128.7. HRMS (ESI): $C_{11}H_{14}N_2O_3$ + Na calc.: 245.12, found: 245.20.

Synthesis of 4a: Compound **Bz-F-OMe** (1 eq., prepared using phenyl acetic acid) was dissolved in methanol (5 mL) followed by dropwise addition of hydroxylamine hydrochloride salt (3 eq.) in potassium hydroxide (5 eq. pH of the reaction medium *ca*. 9) and methanol (10 mL) as a solvent at 0 ºC. This mixture was stirred for 8 h at room temperature. Conversion was monitored by TLC ($CHCl_3$:CH_3OH = 95:05 v/v). Then methanol was removed by rotary evaporator and the reaction mixture was washed with cold water to remove the excess salt. Subsequently, the precipitate was washed with ethyl acetate (10 ml x 3) with constant stirring to remove the unreacted starting material. Finally, the precipitate was washed with diethyl ether (7 ml) once. The obtained compound **4a** was purified by column chromatography (silica gel 100-200 mesh, eluent: 5% methanol in chloroform). The product was isolated as a off-white solid with 72% yield. (m.p. 134-138 °C). ^{1}H NMR (400 MHz, DMSO-d6):δ 2.90-2.95 (2H, 2.92 (d, J = 6.7 Hz), 2.92 (d, J = 6.7 Hz)), 3.75-3.76 (2H, 3.75 (s), 3.75 (s)), 4.87 (1H, t), 7.18-7.40 (10H, 7.30 (tt), 7.29 (dddd), 7.23 (dddd), 7.24 (tt), 7.32 (dddd), 7.35 (dddd). ^{13}C NMR (100 MHz, DMSO-d6): δ(ppm) 128.92, 128.71,

173.89, 42.5, 137.11, 167.26, 134.8, 38.37, 28.71, 128.71, 129.30, 129.30, 129.11, 129.11, 53.61. HRMS (ESI): $C_{17}H_{18}N_2O_3$+Na calc.: 321.1314, found: 321.1419.

Synthesis of 4b: Compound **Bz-F-OMe** (1 eq.) was dissolved in methanol (15 mL) followed by dropwise addition of aqueous solution of potassium hydroxide (2 eq., pH of the reaction medium *ca*. 9) at 0 ºC. This mixture was stirred for 12 h at room temperature after which it was acidified to pH *ca*. 2 using dil. HCl. Then methanol was removed by rotary evaporator and reaction mixture was washed with cold water. A white colored solid was obtained in 92% yield. Further, the crude compound **4b** was purified by column chromatography (silica gel, mesh 100-200, eluent phase 5% CH_3OH in $CHCl_3$). A white product (88% yield, m.p. 121-125 °C) was obtained at this stage. ^{1}H NMR (400 MHz, DMSO-d6): δ2.87-2.91 (2H, 2.89 (d), 2.89 (d), 3.75-3.76 (2H, s), 4.72 (1H, t), 7.16-7.40 (10H, 7.35 (dddd), 7.32 (dddd), 7.25 (dddd), 7.25 (dddd), 7.19 (tt), 7.30 (tt), ^{13}C NMR (100 MHz, DMSO-d6): δ(ppm) 128.92, 136.25, 55.99, 176.55, 173.89, 129.11, 128.71, 42.5, 129.30, 128.71, 38.92, 134.8. HRMS (ESI): $C_{17}H_{17}N_2O_3$ + Na calc.: 306.1210, found: 306.1311.

Synthesis of 4c: L-Phenylalanine hydrochloride methyl ester (1.49 g, 1 eq., 6.95 mmol) was taken in dry THF (15 mL), and triethylamine (1.5 mL, 1.5 eq., 10.42 mmol) was added dropwise to it at 0 ºC followed by stirring for 10 min. In a separate flask, 1-NAA (1.42 g, 1.1 eq., 7.64 mmol) and CDI (1.69 g, 1.5 eq., and 10.42 mmol) were taken in dry THF (15 mL) and stirred for 15 mins. This activated acid solution was added dropwise at 0 ºC to the basic mixture of L-Phenylalanine hydrochloride methyl ester followed by stirring under N_2 atmosphere for 1.5 h at RT. The reaction was monitored by TLC (eluent phase was 5:95 v/v MeOH:$CHCl_3$). THF was removed from the reaction mixture under vacuum, and 30 ml of $CHCl_3$ was added to it. The $CHCl_3$ layer was washed with dil. HCl and sat. $NaHCO_3$, dried over sodium sulphate. $CHCl_3$ removal by rotary evaporator yielded crude product that was purified by column chromatography (stationary phase was silica 100-200 mesh, and eluent phase was $CHCl_3$). Obtained 2.2 g (91%) as white solid, m.p. 125 ºC. FTIR (KBr pellet, cm^{-1}): 1751 (ester, C=O str.), 1647 (amide, C=O str.), 1535 (N-H bend), 3305 (N-H str). ^{1}H NMR (400 MHz, $CDCl_3$, ppm): 2.90-2.92 (2H, Ar-CH_2), 3.95 (3H, COOMe), 3.96-4.11 (2H, Nap-CH_2), 4.83-4.88 (1H, CHCOO), 5.75 (1H, NH), 6.56-7.98 (12H, Ar-H). HRMS (ESI): $C_{22}H_{21}NO_3$+Na calc.: 370.1414, found: 370.1415.

Synthesis of 4d: Compound **4c** (1 g, 1 eq, 2.75 mmol) was dissolved in methanol (15 mL) followed by drop wise addition of 1 N NaOH (6.9 mL, 1.2 eq, 6.9 mmol) at 0 °C. This mixture was stirred for 3 h at

room temperature. Hydrolysis was monitored by TLC. Methanol was removed by rotary evaporator and reaction mixture was acidified by 1 N HCl to pH 2. A white colour solid was formed which was separated by simple filtration and dried over-night under vacuum desiccator to obtain 1.79 g (93%) of **4d**, m.p. 157 °C. ^{1}H NMR (0.1 N NaOH in D_2O): d= 2.77-2.99 (2H, Ar-CH_2), 3.95 (2H, Np-CH_2), 4.36-4.40 (1H, CHCOO), 6.84-7.92 (12H, Ar-H). ^{13}C NMR (100 MHz, 0.1 N NaOD in D_2O) d= 177.94 (CO-Acid) 173.40 (CO-Nap) 55.97 (CH), 39.92 (C-NAP), 37.42 (C-ph), 137.18-123.36 (C-Aromatic). HRMS (ESI): $C_{21}H_{19}NO_3$ + Na cacl: 356.1257, found: 356.1256.

Synthesis of 4e: Compound **4c** (1 g, 1 eq.) was dissolved in methanol (15 mL) followed by dropwise addition of hydroxylamine hydrochloride salt (3eq.) in potassium hydroxide (5eq., pH~8) and methanol (50 mL) as a solvent at 0 ºC. This mixture was stirred for 12 h at room temperature. Conversion was monitored by TLC (chloroform: methanol = 94:06 v/v). Then methanol was removed by rotary evaporator and reaction mixture was washed with cold water and ethyl acetate three times with constant stirring to remove the unreacted starting material and then washed with diethyl ether and then decanted. A white colored solid was obtained which was again washed with ethyl acetate, centrifuged and dried overnight under vacuum desiccator to obtain 1.79 g (34%) of **4e**, m.p. 196 ºC. FTIR (KBr pellet, cm^{-1}): 2500-3300 (br., O-H str.), 1708 (acid, C=O str.), 1662 (amide, C=O str.), 1539 (N-H bend) 3282 (N-H str.). ^{1}H NMR (400 MHz, DMSO-d_6-CD_3OD 9:1 v/v): δ(ppm) = 2.77-2.99 (2H, Ar-CH2), 3.95 (2H, Nap-CH_2), 4.36-4.40 (1H, CHCOO), 6.84 - 7.92 (12H, Ar-H), 10.5-11.0 (1H, OH). ^{13}C NMR (100 MHz, DMSO-d6) d= 170.15 (CO-NHOH), 168.33 (CO-NHPhe), 55.97 (CH), 39.92 (CH_2-Nap) 37.42 (CH_2-Ph) 137.18-123.36 (C-Aromatic). HRMS (ESI): $C_{22}H_{21}NO_3$ calc.: 347.1414, found: 347.1505. $[\alpha]_D^{20}$ = - 29.8 (c = 0.060 wt% in DMF).

A similar protocol was followed for preparing **4f** by employing the corresponding D-Nap-F-OMe precursor in place of **4c**.

Synthesis of 4g: Compound was synthesized using general procedure and reaction was monitored by thin layer chromatography (6% methanol in chloroform). After completion of reaction, crude product was purified by column chromatography. ($CHCl_3$:CH_3OH, 9.4:0.6). A white solid was obtained with 66 % yield. ^{1}H NMR (400 MHz, DMSO-d6): δ 0.85-0.89 (6H, dd), 2.08 (1H, dsept), 3.89-3.90 (2H, s), 4.20 (1H, d), 7.39-7.60 (3H, Ar-H) 7.85-7.96 (3H, Ar-H).^{13}C NMR (100MHz, DMSO-d6) δ(ppm) 175.7,

122.91, 57.42, 126.70, 130.23, 41.29, 31.44, 135.10, 29.75, 38.30, 126.3, 128.2, 167.26, 18.62, 18.62, 139.24, 126.80. HRMS (ESI): $C_{17}H_{20}N_2O_3$+Na calc.: 323.3514, found: 323.3610.

Synthesis of 4h: Compound was synthesized using general procedure and after completion of reaction, was purified by column chromatography. ($CHCl_3$:CH_3OH, 9.4:0.6 v/v). Product was isolated as a white solid was obtained with 66 % yield. ^{1}H NMR (400 MHz, DMSO-d6) : δ 0.80-0.91 (6H, 0.83 (t), 0.88 (d), 1.16-1.24 (2H, qd), 1.92 (1H, dqt), 3.89-3.90 (2H, 3.90 (s), 3.90 (s)), 4.30 (1H, d), 7.39-7.60 (3H, ddd), 7.46 (ddd), 7.44 (m), 7.65 (1H, ddd), 7.85-7.96 (3H, (dddt). ^{13}C NMR (100 MHz, DMSO-d6) δ(ppm) 133.47, 131.83, 25.14, 126.89, 41.19, 126.66, 36.53, 11.50, 173.89, 123.67, 167.26, 131.99, 57.42, 129.09, 128.37, 126.56134, 14.48, 125.88. HRMS (ESI): $C_{18}H_{22}N_2O_3$+Na cacl: 337.3857, found: 337.3907.

Synthesis of 4i: Compound **4i** was synthesized using general procedure and after completion of reaction, was purified by column chromatography using $CHCl_3$:CH_3OH, 9.4:0.6 v/v as eluent. A white solid was obtained with 66 % yield. ^{1}H NMR (400 MHz, DMSO-d6) : δ 0.80-0.91 (6H, 0.83 (t), 0.88 (d), 1.16-1.24 (2H, qd), 1.92 (1H, dqt), 3.89-3.90 (2H, 3.90 (s), 3.90 (s)), 4.30 (1H, d), 7.39-7.60 (3H, ddd), 7.46 (ddd), 7.44 (m), 7.65 (1H, ddd), 7.85-7.96 (3H, (dddt). ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 133.47, 131.83, 25.14, 126.89, 41.19, 126.66, 36.53, 11.50, 173.89, 123.67, 167.26, 131.99, 57.42, 129.09, 128.37, 126.56134, 14.48, 125.88. HRMS (ESI): $C_{18}H_{22}N_2O_3$+Na cacl: 337.3857, found: 337.3907.

Synthesis of 4j: Compound **4j** was synthesized using general procedure and after completion of reaction, was purified by column chromatography. ($CHCl_3$:CH_3OH, 9.4:0.6). A white solid was obtained with 66 % yield. ^{1}H NMR (400 MHz, DMSO-d6): δ 1.38 (3H, d), 2.32-2.57 (4H, 2.55 (d), 2.47 (ddd), 2.37 (ddd), 2.55 (d), 3.46 (1H, tdd), 4.36 (1H, q), 5.54 (1H, ddd), 6.74 (1H, d), 7.07-7.20 (3H, 7.15 (ddd), 7.10 (ddd), 7.12 (td), 7.24 (1H, ddd). ^{13}C NMR (100 MHz, DMSO-d6) δ(ppm) 130.23449, 50.09, 126.80554, 167.26751, 128.2, 175.7, 126.70151, 38.30882, 17.5, 122.91848, 135.1079, 41.29716, 126.3, 139.24721, 29.75592. HRMS (ESI): $C_{15}H_{16}N_2O_3$+Na cacl: 295.3017, found: 295.3112

Suberanilic Acid[2]: Freshly distilled aniline (4.09 g, 0.044 mol) and suberic acid (6.96 g, 0.040 mol) were combined in a large test tube and heated at 185-190°C for 10 min. Vigorous bubbling evolution of water from the melt was evident. This was cooled and dispersed in a solution of 4.0 g of KOH in 50 mL of water with stirring for 20 min. The resulting white suspension was suction filtered. The solid was rinsed with water and dried to afford 1.87 g (12 %) of suberic dianilide, mp 183-185 °C (mp= 184-186°C). The clear

filtrate was acidified with aqueous HCl. The resulting heavy white precipitate was filtered, and the solid was stirred with 100 mL of water at 50 °C. This was filtered hot, and the solid was stirred in another 80 mL of hot water, filtered, rinsed with hot water, and dried, giving 4.16 g (47%) of white solid: mp 124-126 °C (mp 125-127 °C); ^{1}H NMR (400 MHz, DMSO-d6) δ 11.97 (s, 1H), 9.84 (s, 1H), 7.57 (d, 2H), 7.26 (t, 2H), 2.27(t, 2H), 2.18 (t, 2), 1.28 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d6) δ(ppm) 174.69, 171.42, 139.56, 128.84 (2C), 123.11, 119.23 (2C), 36.56, 33.82, 28.61, 28.64, 25.17, 24.59.

Methyl Suberanilate[2]: Suberanilic acid (4.14 g, 0.0166 mol) was dissolved in 25 Ml of methanol, and 0.5 g of Dowex 50W-X2 acid resin was added. This was heated at reflux with magnetic stirring for 22 h, cooled, filtered, and evaporated to afford a pale yellow solid. The solid was dissolved in 10 mL of chloroform (markedly endothermal dissolution) and passed through a 2 x 3 cm column of silica gel, rinsing with additional chloroform. Evaporation afforded 4.10 g (94% yield) of methyl suberanilate.

Suberanilohyroxamic Acid (SAHA)[2]: Hydroxylamine hydrochloride (2.17 g, 0.0312 mol) was dissolved in 15 mL of methanol in a 50 mL flask equipped with magnetic stirring and an addition funnel. One milligram of phenolphthalein was added. A solution of sodium metal (1.08 g, 0.0468 mol) in 15 mL of methanol was placed in the addition funnel and enough added to reach a pink end point. A precipitate of NaCl appeared. Solid methyl suberanilate (4.10 g, 0.0156 mol) was added, which dissolved readily. The remainder of the sodium methoxide solution was added, and the mixture was stirred for an hour. After a few hours, a thick precipitate had appeared. This was left for a total of 24 h at room temperature and then rinsed into 100 mL of water where most of it dissolved. Glacial acetic acid (4.0 g) was added with stirring. The resulting heavy precipitate was suction filtered, rinsed with water, then slurried with another 75 mL of water, filtered, and rinsed again. The solid was dried at room temperature, affording 3.70 g (90%) of white solid, mp 159-160.5 °C, ^{1}H NMR (400 MHz, DMSO-d6) δ 10.33 (s, 1H, 9.84 (s, 1H, 8.66 (s, 1H), 7.57 (d, 2H), 7.27 (t, 2H), 7.00 (t, 1H), 2.27 (t, 2H), 1.92 (t, 2H), 1.47 (q, 2), 1.26 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d6) δ 171.45, 169.34, 139.58, 128.87 (2C), 123.14, 119.26 (2C), 36.61, 32.48, 28.65 (2C), 25.27 (2C).

Histone Deacetylase (HDAC) assay and IC_{50} calculation: *In vitro* HDAC inhibition was performed using BPS Bioscience human recombinant HDAC1 (#50051), HDAC2 (#50002), HDAC3 (#50003), HDAC6 (#50006), HDAC10 (#50060), and fluorogenic HDAC substrate 3 (#50037) in HDAC assay buffer (#50031) and HDAC assay developer (#50030) following the protocol of Gryder, B.E. et al.[4] In

brief, all the compounds were dissolved in DMSO. Different dilutions of the compounds (10, 50, 100, 200, 500, and 1000 nM) were prepared with 10% DMSO in HDAC assay buffer. 5 µL of diluted compounds were added to a 50 µL reaction mixture to achieve a final concentration of DMSO 1% in all of the reactions. The enzymatic reactions were conducted in triplicate at 37 °C for 30 min in a 50 µL mixture containing HDAC assay buffer, 5 µg of BSA, HDAC substrate (20 µM), HDAC enzyme (HDAC1, HDAC2, HDAC3, HDAC6 or HDAC10), and various concentrations of each compound. After enzymatic reactions, 50 µL of 2x HDAC Developer was added to each well and the plate was incubated at room temperature for an additional 15 min. Fluorescence intensity was measured at an excitation of 360 nm and an emission of 450 nm using a BioTek Synergy microplate reader. The fluorescent intensity data were analyzed using Origin 9.0 software. The percent activity of each compound was calculated according to the following equation:

$$\text{Activity (\%)} = (F - F_b) / (F_t - F_b)$$

Where, F= the fluorescent intensity in the presence of compound, F_t= the fluorescent intensity in absence of the compound (defined as 100% activity), F_b= the fluorescent intensity the absence of HDAC (defined as 0% activity).

Molecular Docking Analysis: *In silico* docking was performed using Autodock Vina[28] run through python to manage the workflow and PyMol to visualize the results. Ligands of compound **4e** and **4f** were prepared by creating an energy-minimized 3D-structure in Chem3D, followed by processing with Autodock Tools 1.5.4 to assign Gasteiger charges, merging nonpolar hydrogens, and set torsional bonds. Initial docking runs were performed within a 40−45 Å cubic search space surrounding the binding pocket of enzymes, with solutions found using an exhaustiveness of **4e** and **4f**, with output modes ranked according to binding affinity (BA). Autodock Vina finds ligand poses with the best fit and strongest BA (global minimums) by a stochastic algorithm to explore surfaces of the rigid molecule, through an iterative series of local optimizations evaluating both intermolecular (hydrophobic interactions, repulsions, hydrogen bonding, etc.) and intramolecular (torsion, rotational torque) factors. The images were generated using the UCSF Chimera software.

Animal Cell Culture: The mammalian cell lines were obtained from the following sources: HEK-293T (ATCC-CRL-3216), MCF-7 (ATCC-HTB-22), MDA-MB-231 (ATCC-HTB-26), A549 (ATCC-CRL-185) and HeLa (ATCC-CCL-2) were cultured in complete DMEM media (DMEM supplemented with

10% fetal bovine serum). The H-157 cells (Sigma (Merck): 07030901), were grown in 1:1 mixture of F12 and complete DMEM media. The cells growth was continued in sterile T-flask or multi-well culture plate at 37 ºC in CO_2 incubator maintaining 5% CO_2 and 95% humidity.

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay: The cells (MDA-MB-231, HEK-293T, MCF-7, Hela and H-157) were seeded at 1 x 10^4 cells per well in a 96 well microtiter plates in complete DMEM and were incubated overnight for the attachment. Further for 24 h, cells in triplicates were treated with different concentrations of the compounds (144, 115, 86, 58, 29 nM) shown in Figure 4. For MTT assay, cells were treated with 20 µL of 5 mg/mL solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) (MP Biomedicals) in 20 mM PBS and after 4 h all of the medium including MTT solution (5 mg/mL) was aspirated from the wells. The remaining formazan crystals were dissolved in 150 µL of DMSO and the absorbance was measured at 540 nm using microplate reader. The cytotoxicity index was determined using the untreated cells as negative control. The percentage of cytotoxicity was calculated using the background-corrected absorbance as follows:

$$\% \text{ cytotoxicity} = \left\{ 1 - \frac{\text{(absorbance of experimental well)}}{\text{(absorbance of negative control well)}} \right\} \text{X } 100$$

Cell cycle study by FACS: MDA-MB-231 and HEK-293T cells (1 x 10^6) were cultured and treated with 115 nM each of **4e** and SAHA for 24 h, were trypsinized and washed twice with PBS by centrifugation at 300g for 5 min at 4 ºC. Cell pellets were re-suspended in 1 ml of 1% paraformaldehyde (PFA) fixation solution (in PBS) and incubated on ice for 1 h. PFA solution was removed by washes and cells were re-suspended in 0.5 ml PBS followed by addition of 4.5 ml cold 70% ethanol dropwise over 30 seconds to 1 min at 4 ºC. After 3 h, ethanol was replaced with PBS washing and cells were re-suspended in 40 µg/ml propidium iodide (PI) staining solution. After 30 minutes of PI staining, PI-stained cells were finally acquired & analyzed by flow-cytometry (BD FACS ARIA III).

Annexin V/ PI apoptosis assay by FACS: The apoptosis assay was performed using the Annexin V-FITC apoptosis assay kit (Sigma-Aldrich, APOAF). In brief, the MDA-MB-231 and HEK-293T cells (1 x 10^6) in 60 mm culture dish, were treated with 115 nM of compound **4e**, SAHA for 24 h, trypsinized and washed twice in PBS at 300*g*, 5 min at 4 ºC. The cells were stained for 10 min with Annexin V-FITC conjugate, and Propidium Iodide (PI) provided in the kit then washed with 20 mM PBS (pH 7.4) and

finally suspended in the PBS. The stained cells were acquired and analyzed by flow-cytometry (BD FACS ARIA III).

Western blot Analysis: (1) MDA-MB-231 and HEK-293T cells were treated with compound **4e** and SAHA (58 nM and 115 nM) for 24 h. Post treatment, cytoplasmic lysates were prepared using epigenetic nuclear extract isolation kit (OP-0002). Equal amounts of total protein were loaded on 12% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane; membrane was then blocked for 1 h at RT with 5% non-fat dried skimmed milk in PBST (0.1% Tween-20 in PBS). Following the manufacturer's recommendations, membranes were probed overnight at 4 ºC with primary antibodies for acetylated α-tubulin (Santa Cruz Biotechnology; 6-11B-1), anti-α-tubulin (DSHB, 12G10) and anti-actin Ab-5 (BD Transduction Laboratory) as a loading control. HRP-conjugated anti-mouse (Genie) secondary antibodies were used at a dilution of 1:10,000 for 1 h at room temperature. Chemiluminescence detection was carried out with the ECL plus Western Blotting Detection System (Thermo Pierce).

(2) The western blot on lysates of MDA-MB-231 and HEK-293T cells treated with **4e** hydrogel were also performed. In brief, MDA-MB-231 and HEK-293T cells treated with **4e** hydrogel (4 mg/ml, volume of gel *ca*. 125 mm^3) for 24 h, 36 h and 48 h. After treatment, samples were prepared and subjected to western blotting following the method mentioned earlier.

Confocal Laser Scanning Microscopy (CLSM): The MDA-MB-231 cells were grown in T-25 flask and treated with 58 nM compound **4e** or SAHA for 12 h and 24 h. Post-treatment cells were washed with PBS (phosphate-buffered saline) and fixed using 100% chilled methanol for 15 min at -20 ºC. Cells were then rehydrated and permeabilized with rehydration buffer (10 mM Tris, 150 mM NaCl, 0.1% Triton X-100) for 5 min and blocked in 5% Normal Goat Serum for 30 min at room temperature. Cells were stained overnight at 4°C with mouse-derived primary antibodies against anti-acetylated α-tubulin (Santa Cruz Biotechnology; 6-11B-1) and anti- α-tubulin (DSHB, 12G10) followed by three 5-min washes each with PBST (PBS + 0.1% Triton X-100). Alexa Fluor 568 Goat anti-Mouse IgG (A-11031, Life technologies) and Alexa Fluor 488 Goat anti-Mouse IgG (A-11001, Life technologies) secondary antibodies were used against acetylated-α-tubulin and α-tubulin antibodies respectively, as described by the supplier. The nucleus was stained by Hoechst 33342 (Invitrogen) and the fluorescence images were obtained on a Multiphoton Confocal system from Carl Zeiss.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): MDA-MB-231 cells were grown in 100 mm tissue culture petri dish and treated with 115 nM compound **4e** for 24 h. After treatment, the total cellular RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The cDNA strand was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase (Thermo Fisher) Primers were designed by using Integrated DNA Technology (IDT). The PCR were performed in a 25 μL buffer solution containing 1 PCR buffer (Agilent Technology), 1.5 mM of $MgCl_2$, 0.3 mM of dNTPs, 0.25 μM of each primer and 2 units of Taq polymerase (Agilent Tech.). Further, we used 100 ng of cDNA as template for PCR amplification of fragments of all HDAC isoforms for different cycles (20 cycles to 30 cycles) to determine the appropriate conditions for obtaining semi-quantitative differences in their expression levels. We carried out the RT-PCR analyses with 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as control to ensure cDNA quality and loading accuracy. Amplified products were analyzed on a 2% agarose gel and stained with ethidium bromide, to assess expression changes and loading accuracy semi-quantitatively.

Three Dimensional (3D) Multi Cellular Tumor Spheroid (MCTS) Cell Culture: The breast carcinoma (MCF-7) cells (10^4 cells/well) were seeded in 1.5% agarose gel coated 6-well plates in DMEM medium supplemented with 10% FBS. The cells were allowed to grow in colonies for 3 days to get spheroid shape. During growth, 50% of the media was carefully exchanged every other day. The Multi Cellular Tumor Spheroid (MCTS) were grown until they reached uniform sizes (diameter in μm) before treatment. Thereafter, the tumor spheres were treated with compound **4e** for 24 h. After the completion of the treatment duration, the morphological changes in the tumor spheres were examined under the Olympic Fluorescence Microscope and calculated using CellsDense software.

Treatment and Measurement of Multicellular Tumor Spheroids (MCTS): The MCTS was treated by various doses of compound **4e** (50, 100, 200 and 500 nM) for different time points. The size of MCTS, before and after treatment, were determined by measuring their cross-sectional area using an automated image analysis macro developed for use with the CellsDense software package provided with the fluorescence microscope for image analysis. The tumor spheroid volume ratio was calculated using the formula: $R = (V_{dayi}/V_{day0}) \times 100\%$. All of the above said studied was done in triplicates.

Propidium Iodide (PI) staining of MCF-7 Spheroids: MCF-7 cells were seeded onto the wells of a 1.5% agarose gel coated tissue culture treated 6-well plate (10^4 cells/well). The seeded cells were then treated with the compound **4e** at 50, 100, 200 and 500 nM concentrations, 20 mM PBS was used as the

positive control, whereas untreated cells were used as negative controls. Both the treated and untreated cells were then washed thrice with PBS and stained with propidium iodide (PI, 4.5 μM Sigma-Aldrich) for 15 min at 37 °C under 5% CO_2−95% atmosphere. Finally, the cells were washed with PBS thrice to remove the excess PI dyes, and images were captured with a 20X and 40X objective in Olympus fluorescence microscope.

Apparent pK_a determination[3]: 40 mg of **4e** was suspended in 10 mL distilled H_2O (~10 mM). A few drops of 0.1 M NaOH were added to the aqueous suspensions of gelator to bring its pH to 12. At this pH, the compound dissolved completely in water to yield a clear solution. Titration was performed by stepwise increments (0.25 mL) of dilute HCl (0.1 M) at 25 °C with constant strong stirring and pH values were recorded after each addition. As a control, distilled water was also titrated as the above-described methodology; that is, 0.1 M NaOH was added to the water to bring the pH of the water to 12, titrated by 0.1M HCl and then pH values were recorded after each addition. A titration curve was plotted to obtain the value of apparent pK_a.

Gelation and Minimum Gelation Concentration (MGC): 5 mg compound (**4e** or **4f**) was weighed in a vial and 1 mL water was added to it. This mixture was heated in a water bath maintained at 100 °C until the solid dissolved in water (approx. 8-10 min). The resulting solution was allowed to cool to room temperature. Hydrogel was considered to have formed with no flow of solvent was observed upon inversion of the vial. In a different protocol, 5 mg compound was taken in a 5 ml glass vial and dissolved in 200 μl of MeOH. A thin film of the compound was prepared by slow evaporation of the MeOH solvent under the continuous flow of nitrogen. Maximum removal of the solvent was ensured by flowing nitrogen for about 5 min into the vial. Subsequently, 2 ml water was added to the vial and the contents were heated using a heat gun. Gelation was observed upon cooling the vial naturally without disturbance to room temperature. In such case, incremental addition of 500 μL of water was added and the process repeated until flow of solvent was observed. By this way, MGC values were calculated from the maximum volume of solution completely entrapped. Similar MGC value was obtained in 10 mM phosphate buffer (pH = 7.2) as well.

Gel-melting temperature (T_m): The "melting" temperature (T_m) of resultant gel in different salt solutions was determined by the 'inverse flow method'. Gelled samples in sealed tubes were inverted and attached to a thermometer near the bulb end. This assembly was plunged in a stirred, room temperature water bath

and the temperature was raised at *ca*. 5 °C·min^{-1} on a hot plate. T_m was recorded as the temperature at which the gel mass fell.

Circular Dichroism (CD) of hydrogels: 1 mg of **4e** or **4f** was dissolved in 1 mL distilled water or 100 mM carbonate buffer (pH 7, 8, 9 or 10) in CD quartz cuvette (path length 10 mm) by heating- cooling process and equilibrated at 22 °C for 4 h to obtain a clear gel. This sample was used directly for measurements of CD signal. Sample concentrations were adjusted to 0.8 mg/mL of gel state, which corresponded to an A_{295} = 0.1. Far-UV CD spectra (wavelength scans) were recorded using a 1 mm path length quartz cell with 0.5 or 1.0 nm band width, 1s data integration time and 100 nm/min scan speeds. Data were averaged over at least three accumulations, blank subtracted and smoothened.

pH-Induced gelation and injectibility: 4e (2 mg/mL) was suspended in water and its pH was raised to pH-9.0 by adding 100 µL of 0.1 M NaOH solution. The solution was further warmed to get clear, free flowing solution. A drop of phenolphthalein solution (as pH-indicator) was added to this solution. This solution was taken in a syringe and dropped into simulated body fluid (SBF, pH 7.2). Immediate gelatinous precipitate was observed.

For obtaining injectable gels, 4 mg/ml concentration of 4e was employed and the heat-cool process was applied to the suspension. Upon gradual and undisturbed cooling, the sol set into a firm gel. The gel was allowed to set further for 12 h at 4 °C before undertaking further studies.

Rheology: The **4e** hydrogel of 2 mg/mL solution in water or 100 mM carbonate buffer (pH 7, 8, 9 or 10) was prepared by heat-cool process. The samples were allowed to equilibrate at 4 °C for 12 h before the rheological measurements were carried out on them on a Rheoplus MCR302 (Anton Paar) rheometer with parallel plate geometry. For the oscillatory shear measurements, a top parallel plate with a 25 mm diameter and 1.0 mm gap distance were used. The hydrogel was further transferred to the bottom plate of the rheometer. The shear modulus (storage modulus, G', and loss modulus, G'') were plotted against % strain from 0.1% to 100 %. Frequency sweep experiment was performed from 0.1 to 100 rad/s at a constant strain of 1 %. The obtained data were processed with rheometer software.

Field Emission Scanning Electron Microscopy (FE-SEM): For SEM, the samples were scooped from the surface of the vial using a spatula. The extracted samples were dried in a vacuum desiccator for 48 h.

These samples were spread on a carbon tape and gold coated for 120 s. The images were recorded on Carl Zeiss (Ultra 55 plus) FE-SEM at an accelerating voltage of 5 kV.

Transmission electron microscopy (TEM): The morphology of samples was examined using FEI TALOS 200S instrument at a working voltage of 200 kV. The samples for TEM analysis were prepared by drop casting a homogeneous dilute dispersion of **4e** or **4f** over a carbon coated 400 mesh Cu grid. For sample preparation, 0.25 mg of powdered samples of **4e** or **4f** were dispersed separately in 2 mL water. 10 μL of this dispersion was drop-cast on carbon-coated copper grid and stained with 0.1% phosphotungstic acid for 20 s. High-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) energy dispersive X-ray spectroscopy (EDS) mapping was carried out at an accelerating voltage of 200 kV.

Leaching of 4e from its hydrogels in Simulated Body Fluid (SBF, pH 7.2): The **4e** hydrogel (4 mg/mL) (500 mm^3) was prepared in 0.02 M PBS (pH 7.2). Separately, simulated body fluid (SBF) was made by dissolving together NaCl (7.996 gm), $NaHCO_3$ (0.350 gm), KCl (0.224 gm), $K_2HPO_4 \cdot 3H_2O$ (0.228 gm), $MgCl_2 \cdot 6H_2O$ (0.305 gm), 1 M HCl (40 mL), $CaCl_2$ (0.278 gm), Na_2SO_4 (0.071 gm), $(CH_2OH)_3CNH_2$) (6.057 gm) in 1 L milli-Q water. For release study, the hydrogel was kept in an SBF solution, and the release of monomeric form of **4e** hydrogelator from hydrogel was monitored at room temperature by periodically analyzing aliquots in UV-vis spectrophotometer in the region of 190-700 nm.

FACS of cells treated by hydrogel form of 4e: In brief, **4e** hydrogel (4 mg/mL) was prepared in (0.02 M PBS, pH 7.2). MDA-MB-231 and HEK-293T cells were grown in 60 mm culture dish till ~70% confluence. After that, cells were scraped from the center of the culture dish and a hydrogel of disc shape (125 mm^3) was kept. After a fixed time-point (6 h, 12 h, 24 h, 36 h, or 48 h), the cells were stained with propidium iodide (40 μg/mL) prepared in PBS and washed twice with PBS. Finally, the fluorescent microscopic analysis was performed using OLYMPUS CellSens Dimension Microscopy.

Propidium Iodide (PI) staining of cells treated by hydrogel 4e: The cells were seeded onto the issue culture treated 6-well plate (~10^4 cells/well). The seeded cells were then treated with the hydrogel **4e** (4 mg/mL), 20 mM PBS was used as the positive control, whereas untreated cells were used as negative

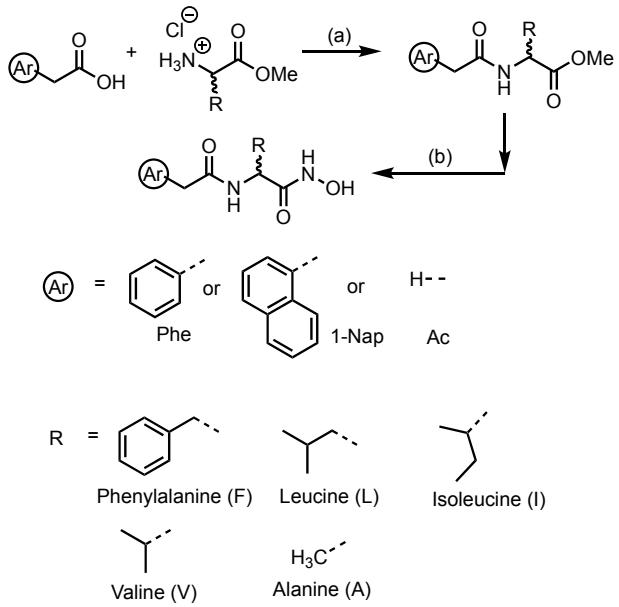


Figure S1a: Synthesis of hydroxamic acid derivatives of amino acids. Reagents and reaction conditions: (a) 1,1'-Carbonyldiimidazole (CDI), Dry THF, 0 °C→RT, N_2 gas, 3-12 h and Et_3N; (b) $NH_2OH.HCl$, KOH, Methanol, 0 °C→RT. The site of linkage is depicted by dashed line.

controls. Both the treated and untreated cells were then washed thrice with PBS and stained with propidium iodide (PI, 4.5 μM Sigma-Aldrich) for 15 min at 37 °C under 5% CO_2−95% atmosphere. Finally, the cells were washed with PBS thrice to remove the excess PI dyes, and images were captured with a 20X objective in Olympus fluorescence microscope.

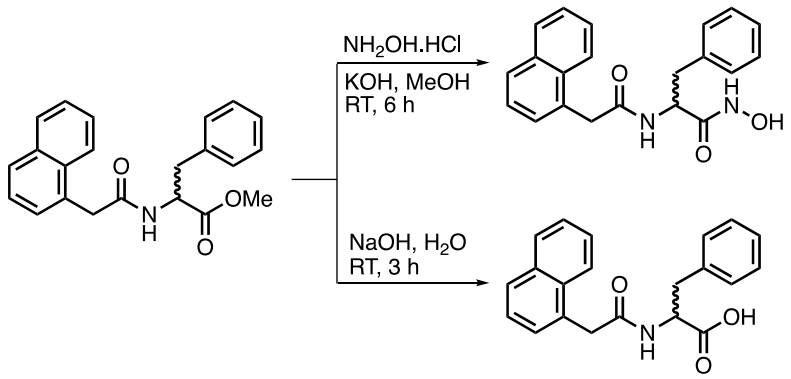


Figure S1b: General synthetic route for the phenylalanine derivatives.

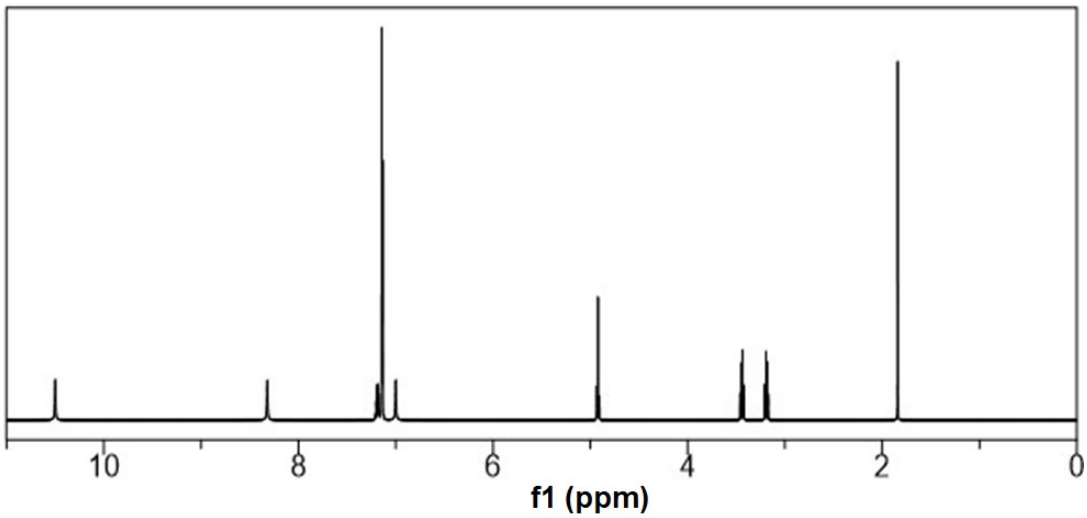


Figure S2a: ^{1}H NMR (400 MHz) spectrum of **2b** in DMSO-d6.

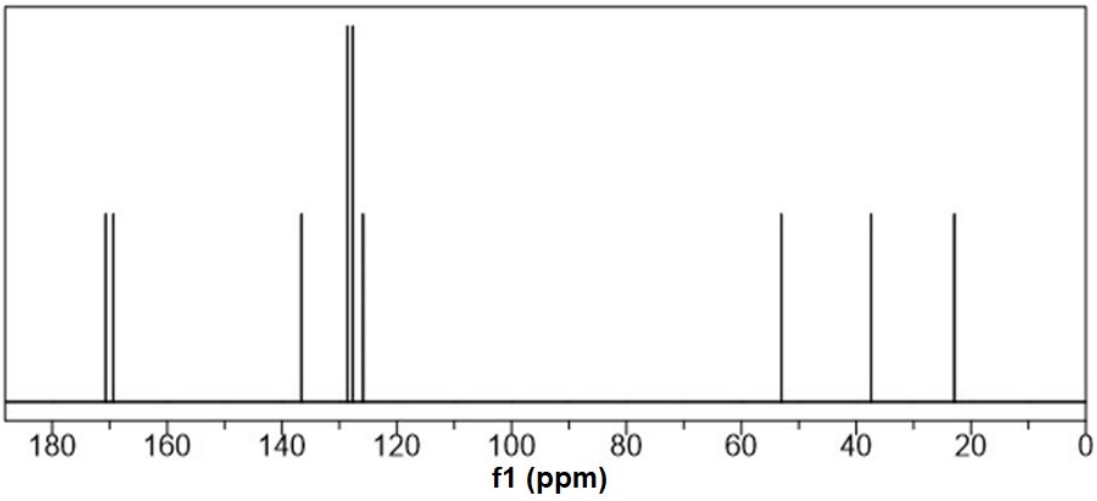


Figure S2b: ^{13}C NMR (100 MHz) spectrum of **2b** in DMSO-d6.

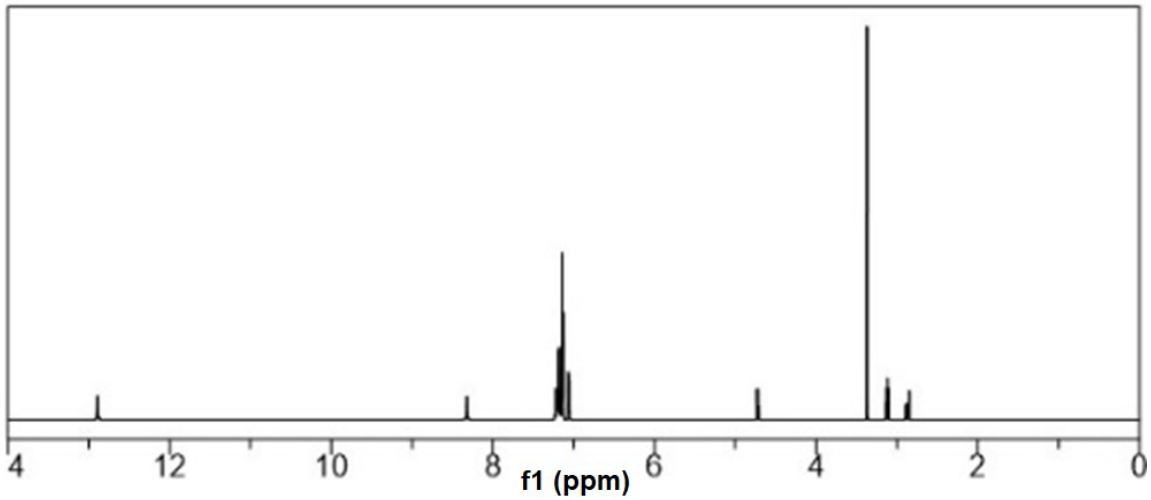


Figure S2c: ^{1}H NMR (400 MHz) spectrum of **4a** in DMSO-d6.

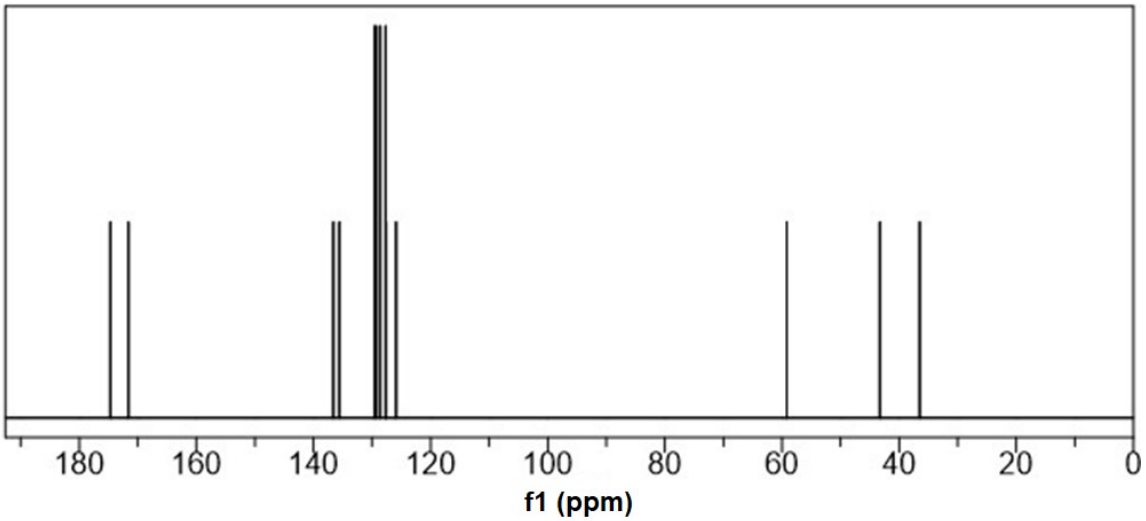


Figure S2d: ^{13}C NMR (100 MHz) spectrum of **4a** in DMSO-d6.

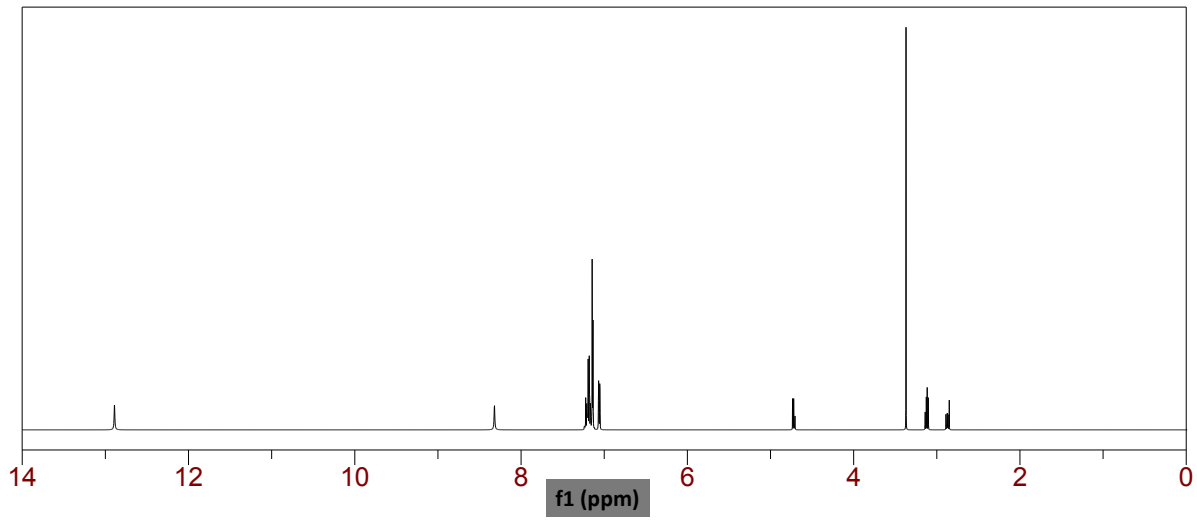


Figure S2e: ^{1}H NMR (400 MHz) spectrum of **4b** in $CDCl_3$.

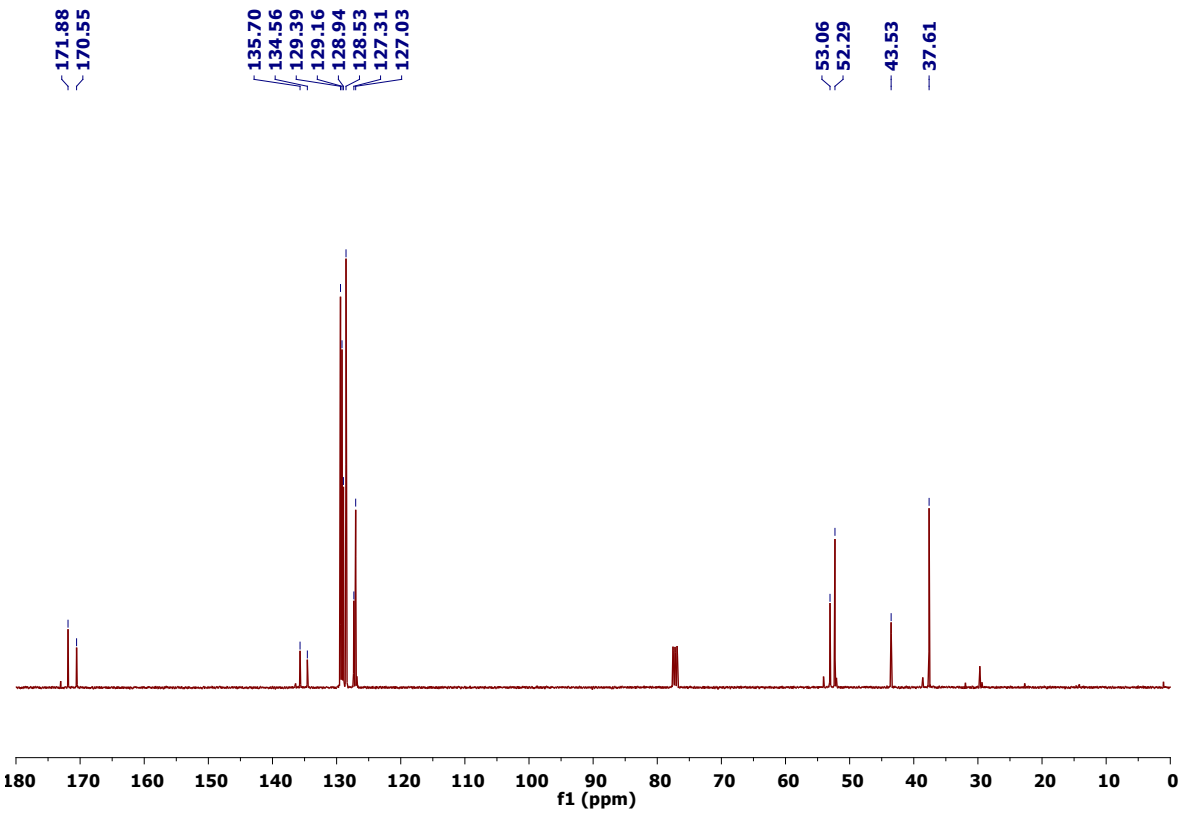


Figure S2f: ^{13}C NMR (100 MHz) spectrum of **4b** in $CDCl_3$.

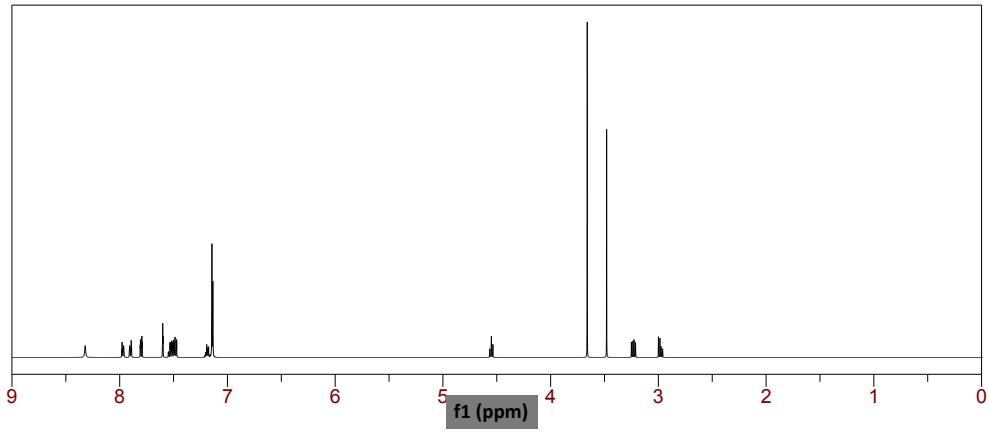


Figure S2g: ^{1}H NMR (400 MHz) spectrum of **4c** in DMSO-d6.

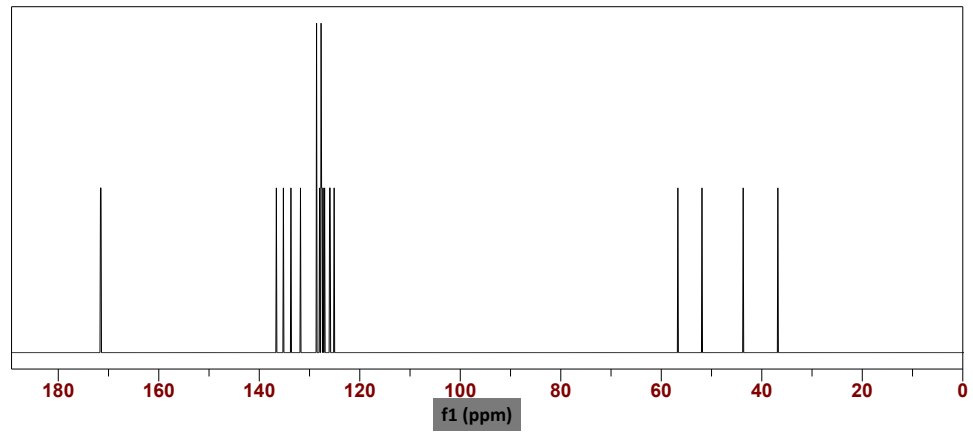


Figure S2h: ^{13}C NMR (100 MHz) spectrum of **4c** in DMSO-d6.

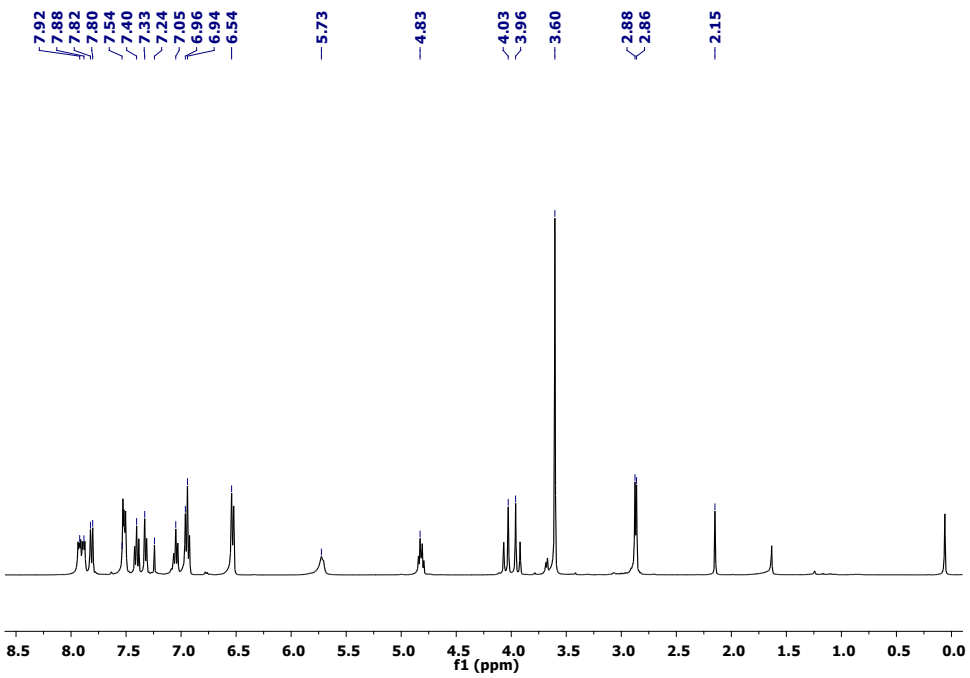


Figure S2i: ^{1}H NMR (400 MHz) spectrum of **4d** in DMSO-d6.

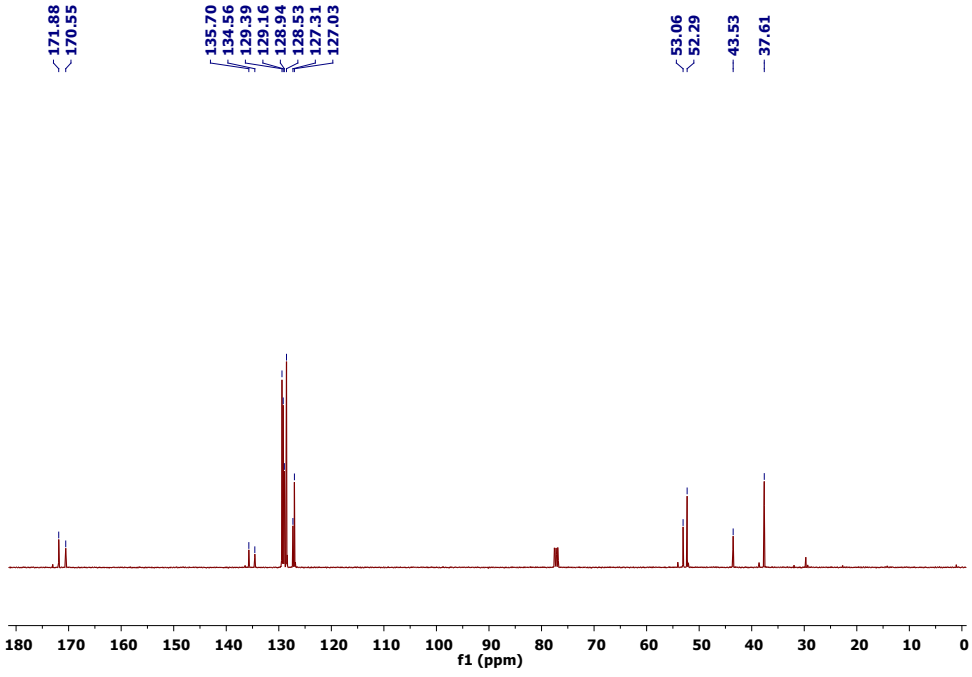


Figure S2j: ^{13}C NMR (100 MHz) spectrum of **4d** in DMSO-d6.

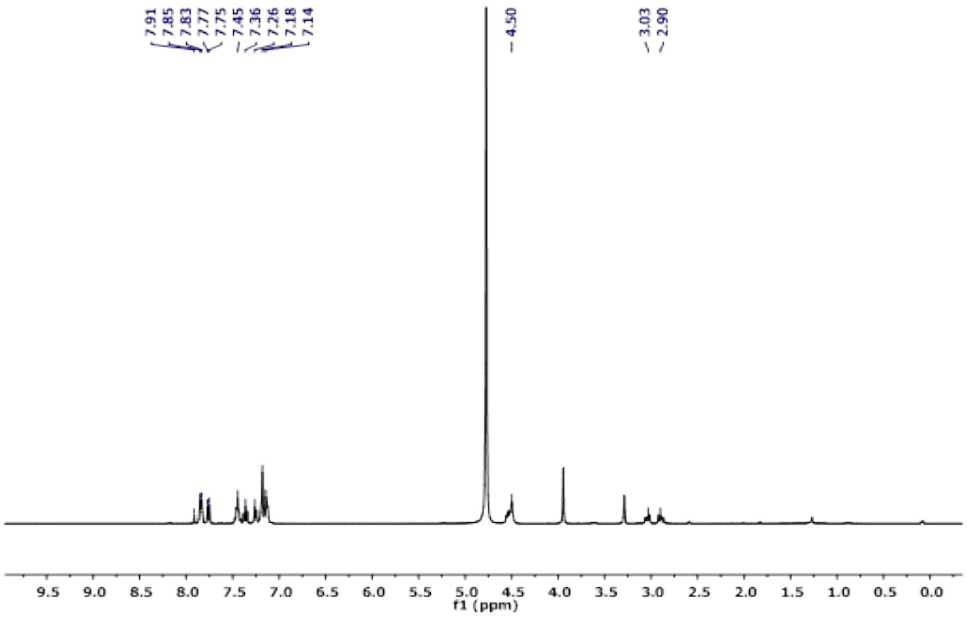


Figure S2k: ^{1}H NMR (400 MHz) spectrum of **4e** in DMSO-d6: CD_3OD (9:1 v/v)**.**

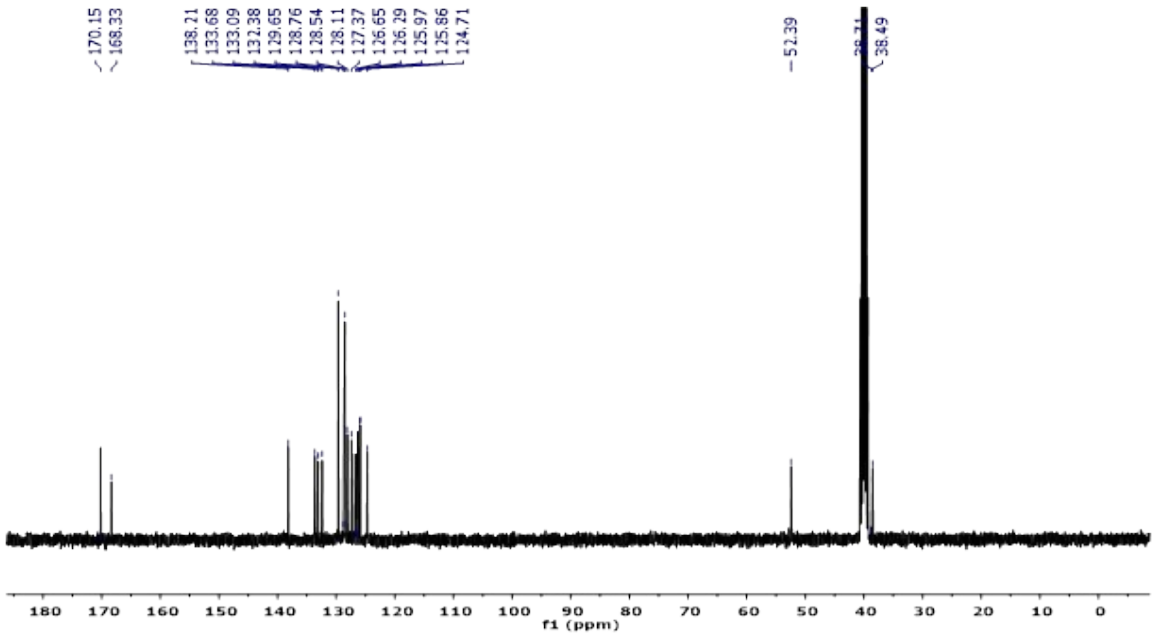


Figure S2l: ^{13}C NMR (100 MHz) spectrum of **4e** in DMSO-d6: CD_3OD (9:1 v/v)**.**

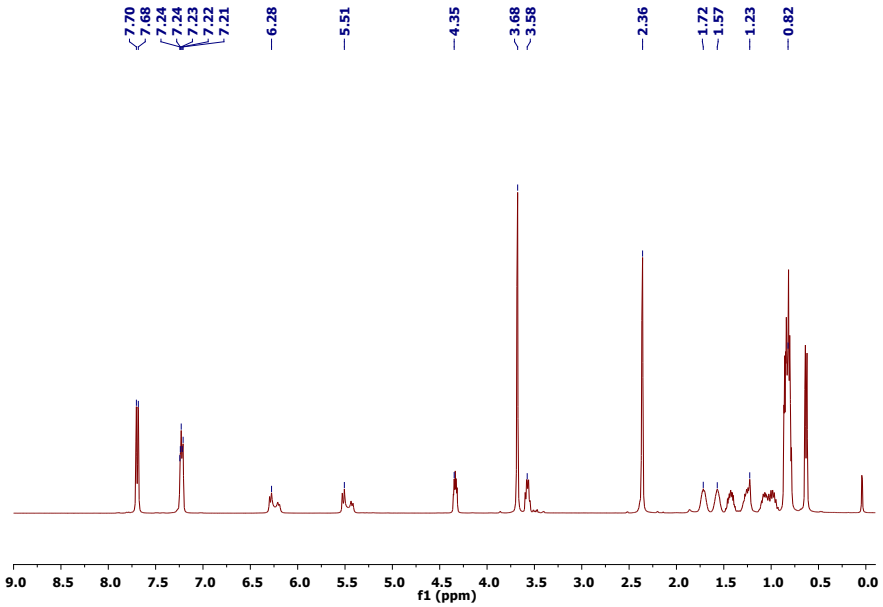


Figure S2m: [1]H NMR (400 MHz) spectrum of **4f** in DMSO-d6.

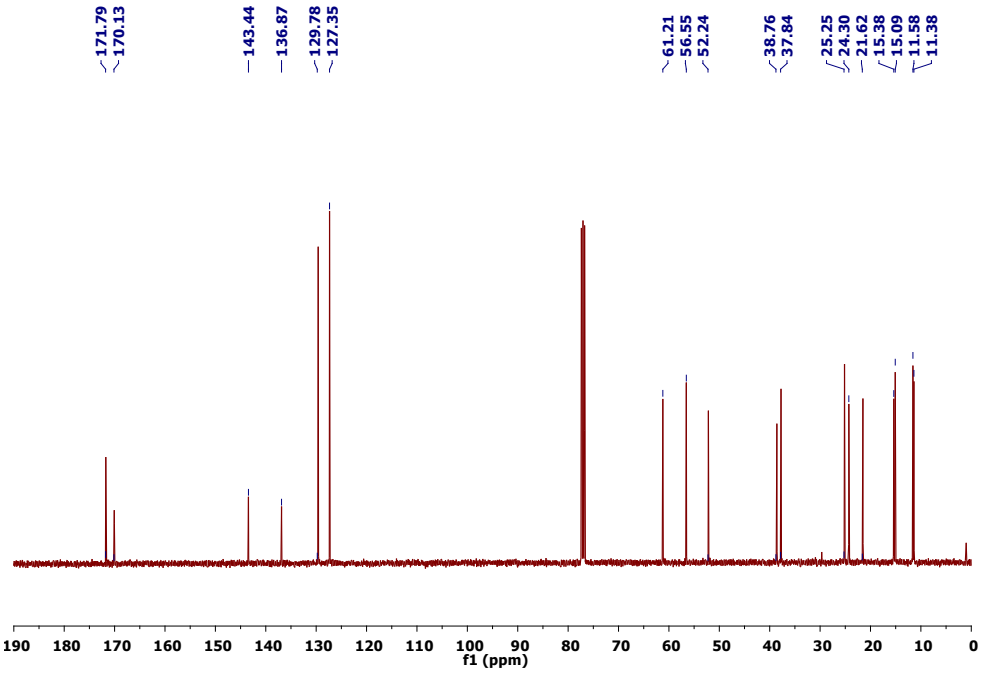


Figure S2n: [13]C NMR (100 MHz) spectrum of **4f** in DMSO-d6.

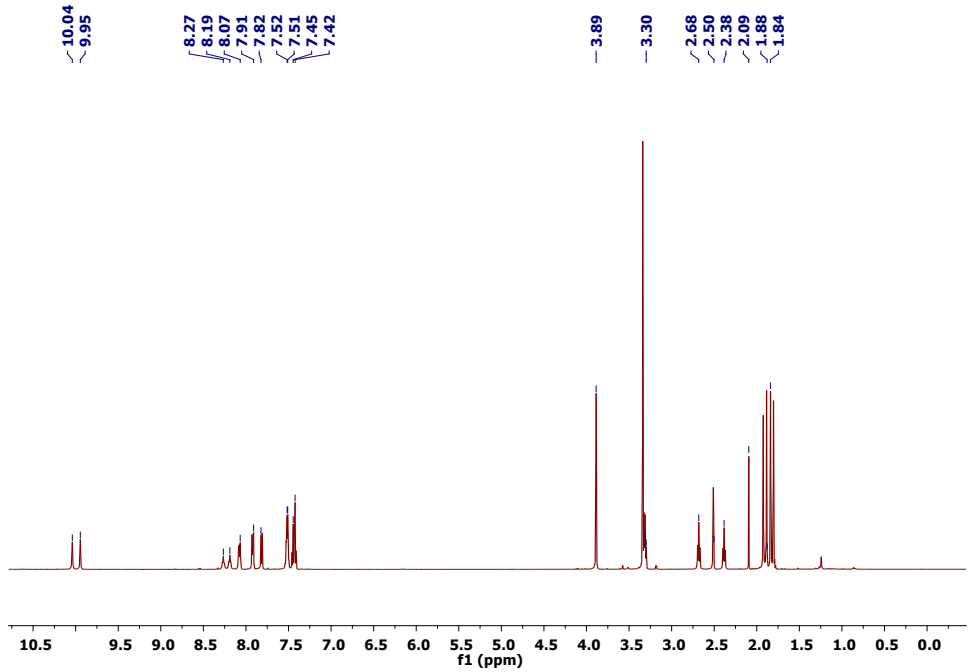


Figure S2o: [1]H NMR (400 MHz) spectrum of **4g** in DMSO-d6.

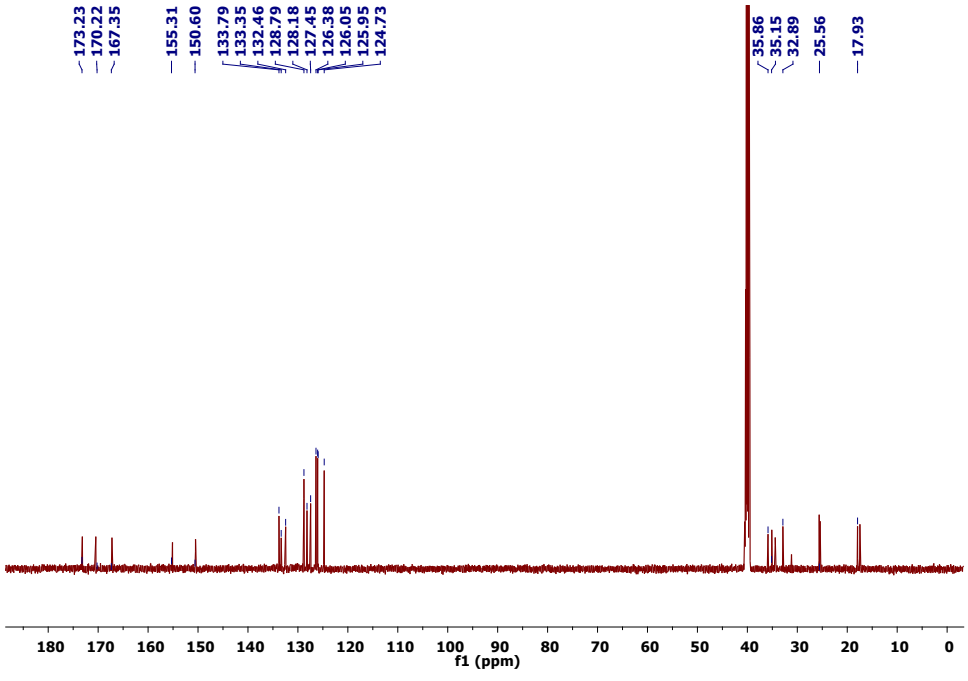


Figure S2p: [13]C NMR (100 MHz) spectrum of **4g** in DMSO-d6.

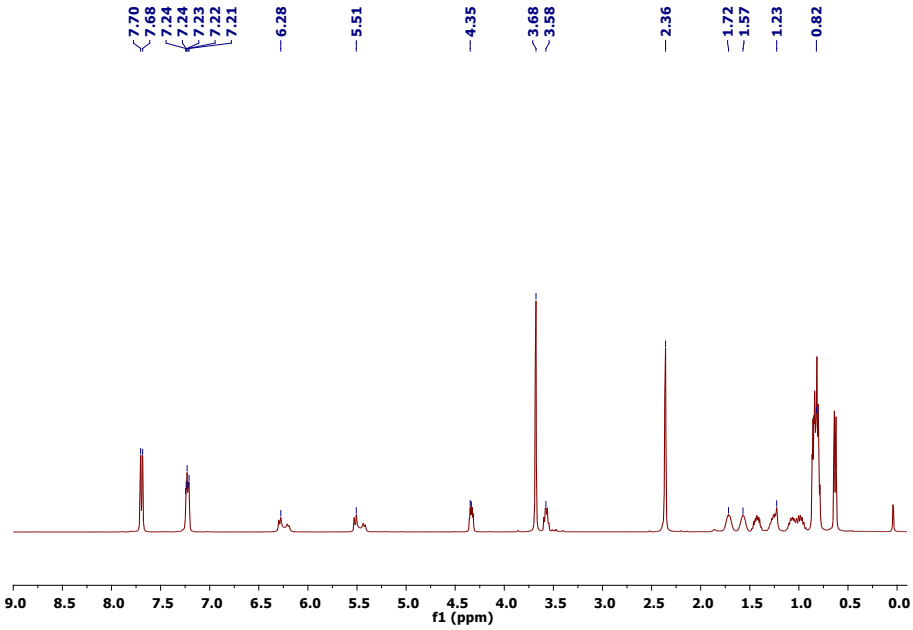


Figure S2q: ^{1}H NMR (400 MHz) spectrum of **4h** in DMSO-d6.

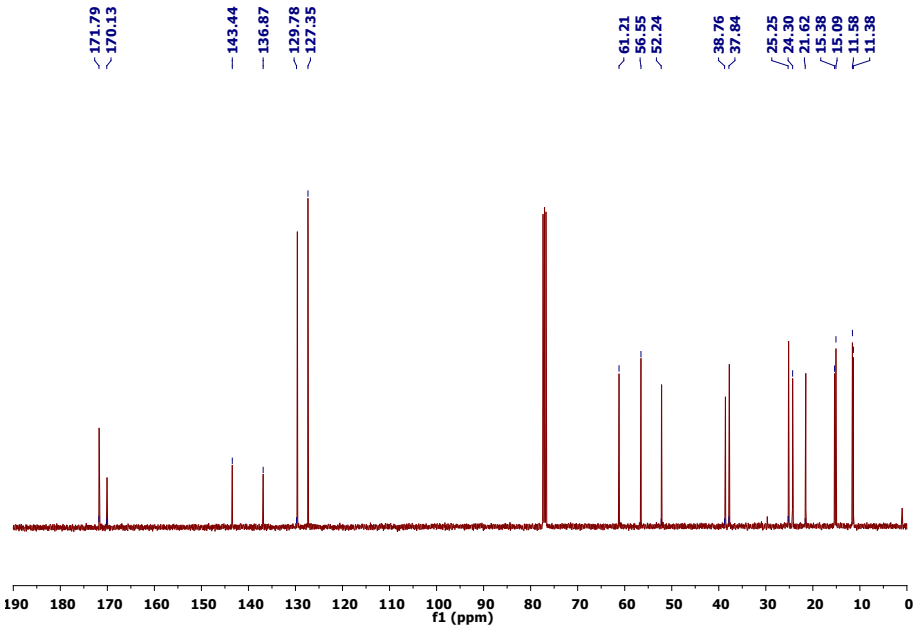


Figure S2r: ^{13}C NMR (100 MHz) spectrum of **4h** in DMSO-d6.

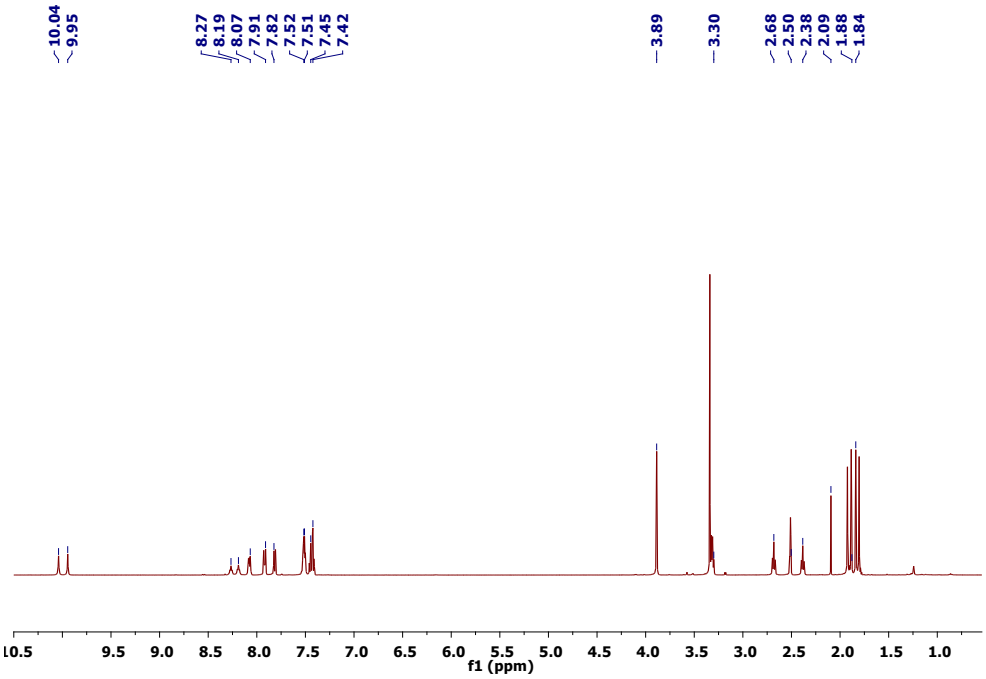


Figure S2s: ^{1}H NMR (400 MHz) spectrum of **4i** in DMSO-d6.

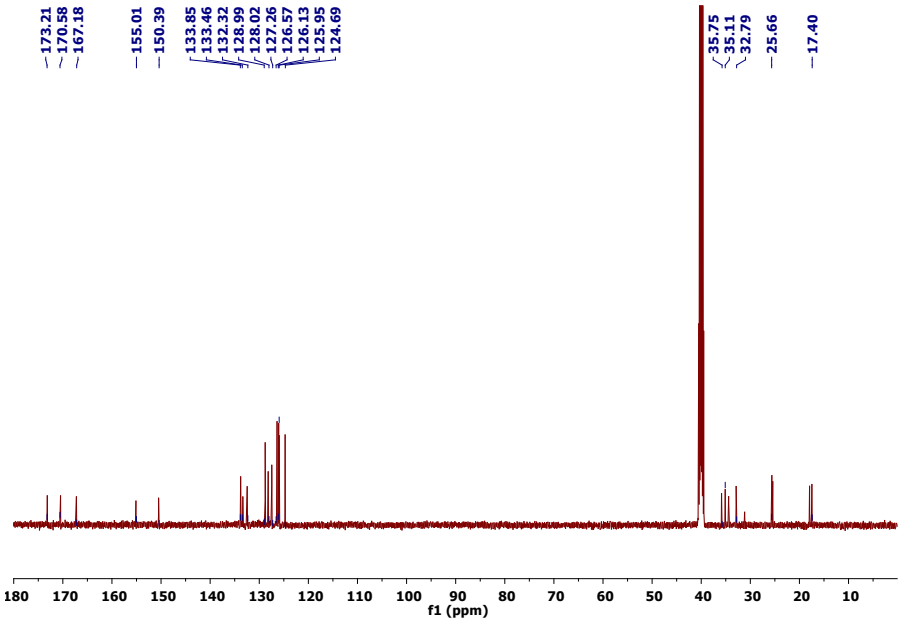


Figure S2t: ^{13}C NMR (100 MHz) spectrum of **4i** in DMSO-d6.

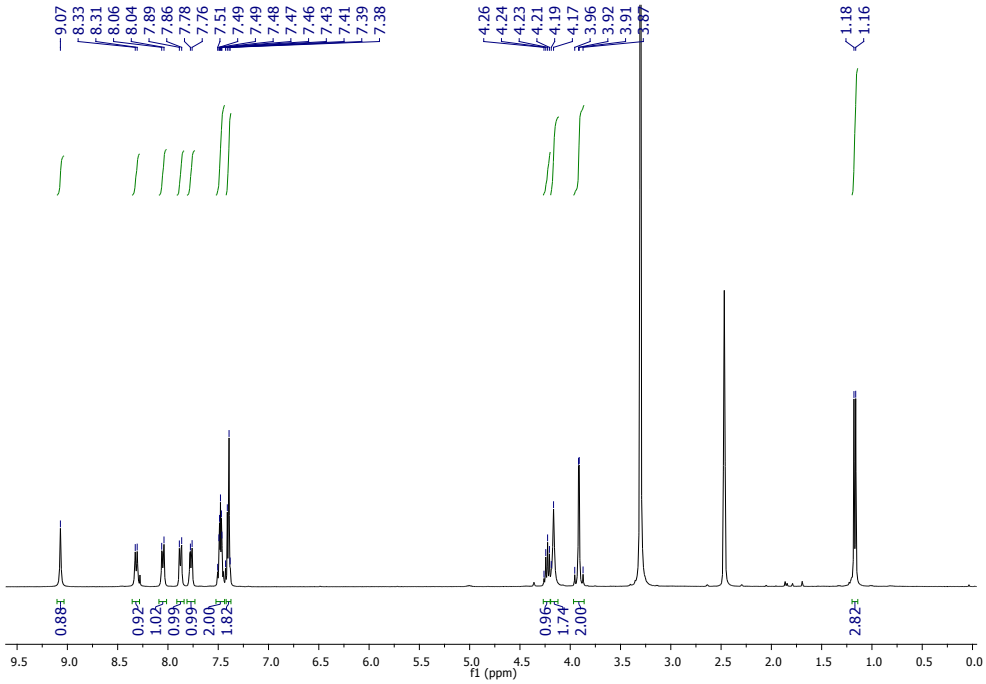


Figure S2u: ^{1}H NMR (400 MHz) spectrum of **4j** in DMSO-d6

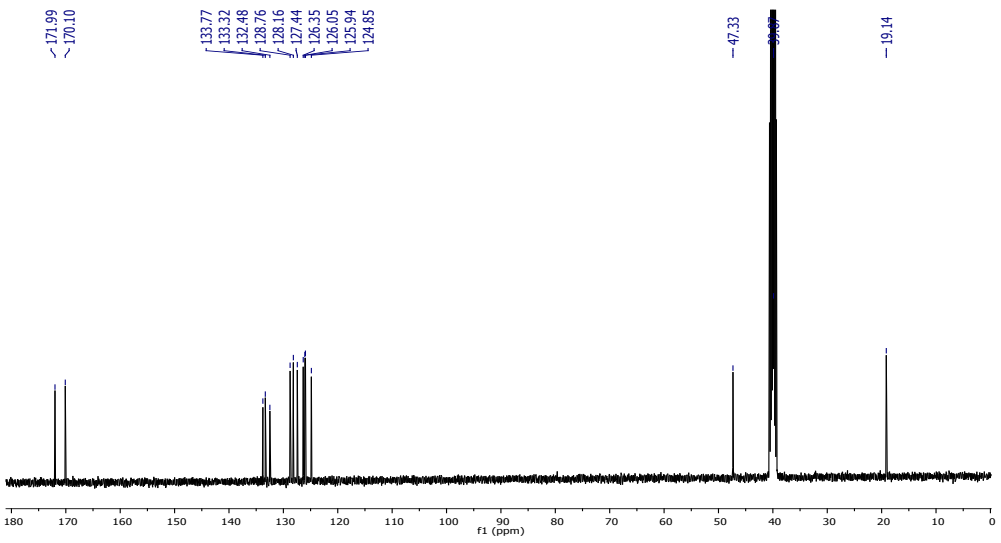


Figure S2v: ^{13}C NMR (100 MHz) spectrum of **4j** in DMSO-d6.

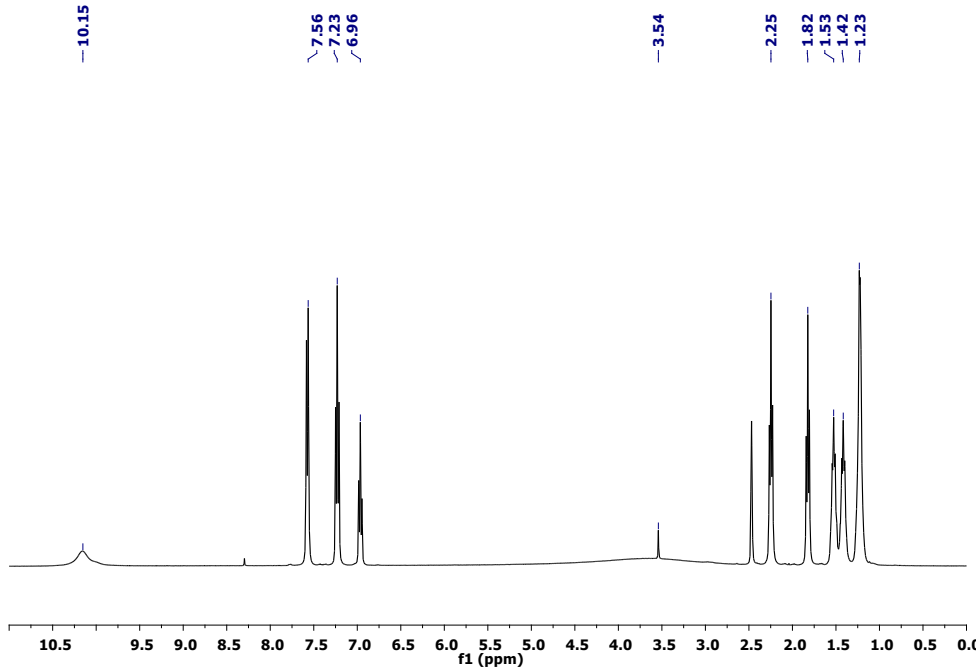


Figure S2w: ^{1}H NMR (400 MHz) spectrum of SAHA in DMSO-d6.

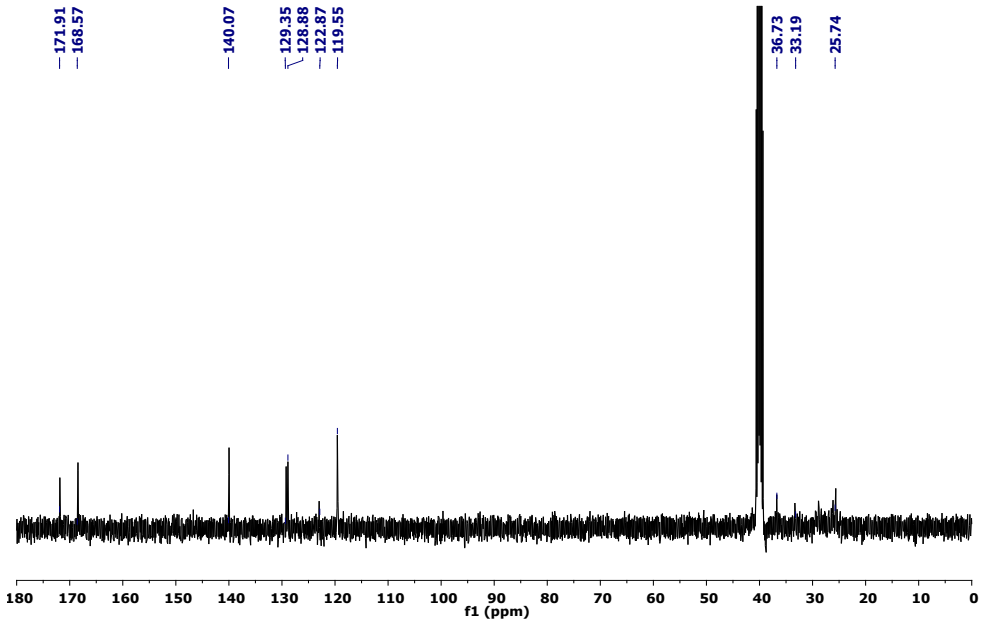


Figure S2x: ^{13}C NMR (125 MHz) spectrum of SAHA in DMSO-d6.

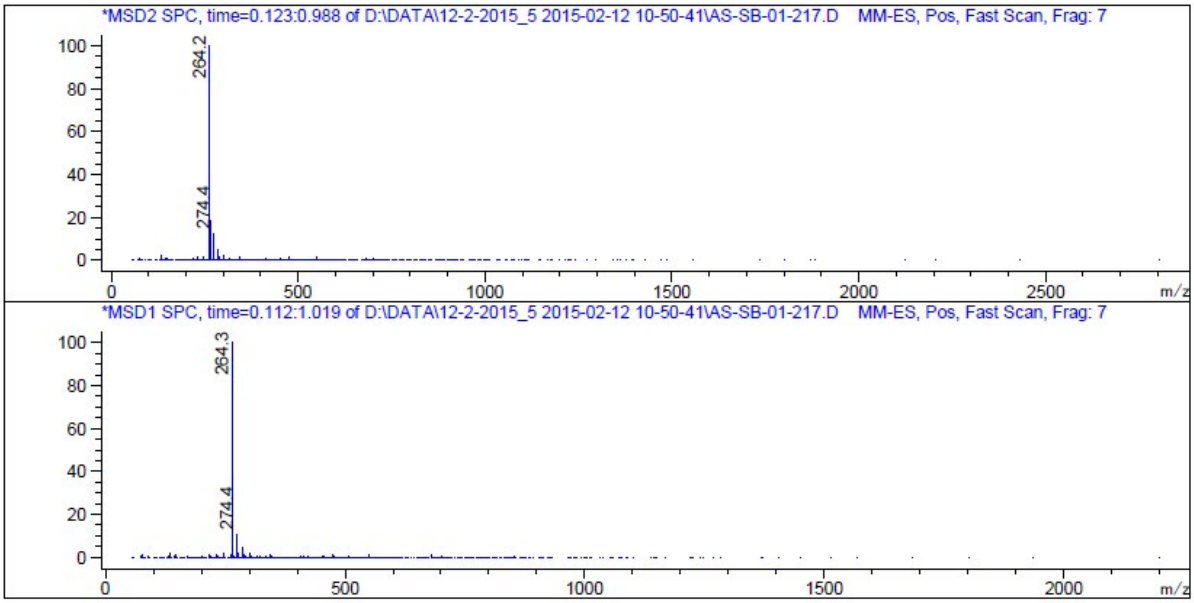


Figure S2y: LR-MS data for the synthesized SAHA.

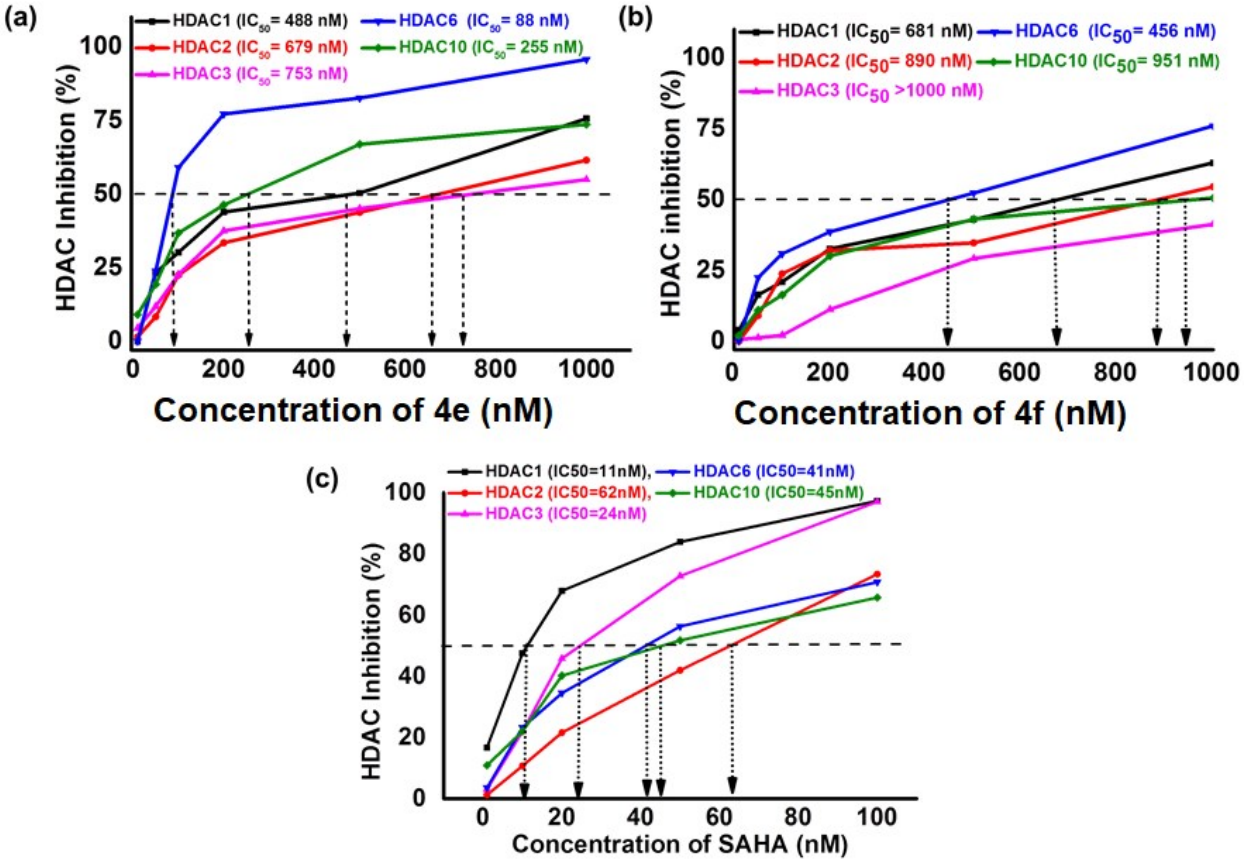


Figure S3a: The IC_{50} (nM) values of a) compound **4e**; b) compound **4f**; and c) SAHA on class I (HDAC1, 2, 3) and class II (HDAC6 and 10) isozymes of HDACs.

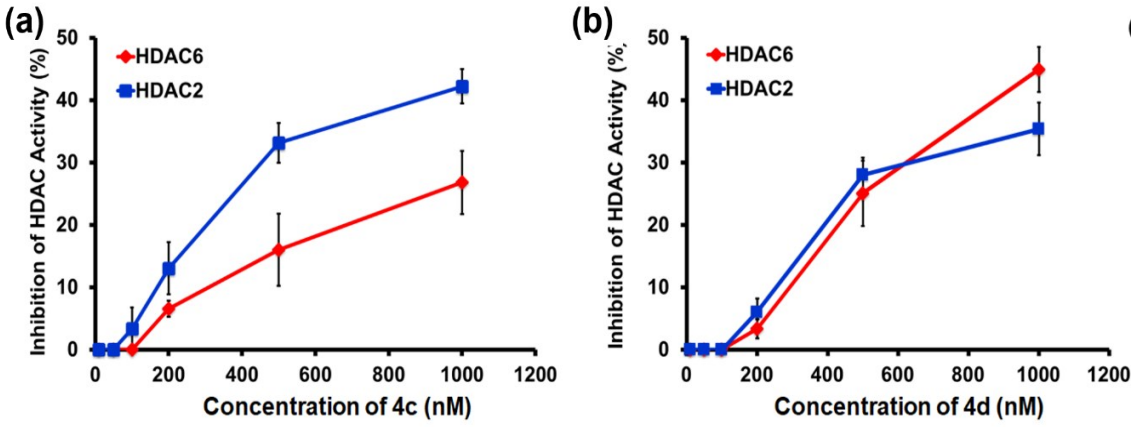
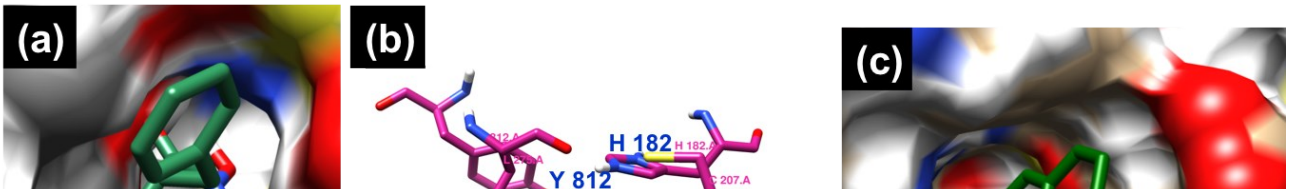


Figure S3b: IC_{50} values of (a) compound **4c**; (b) compound **4d** on HDAC2 and HDAC6.



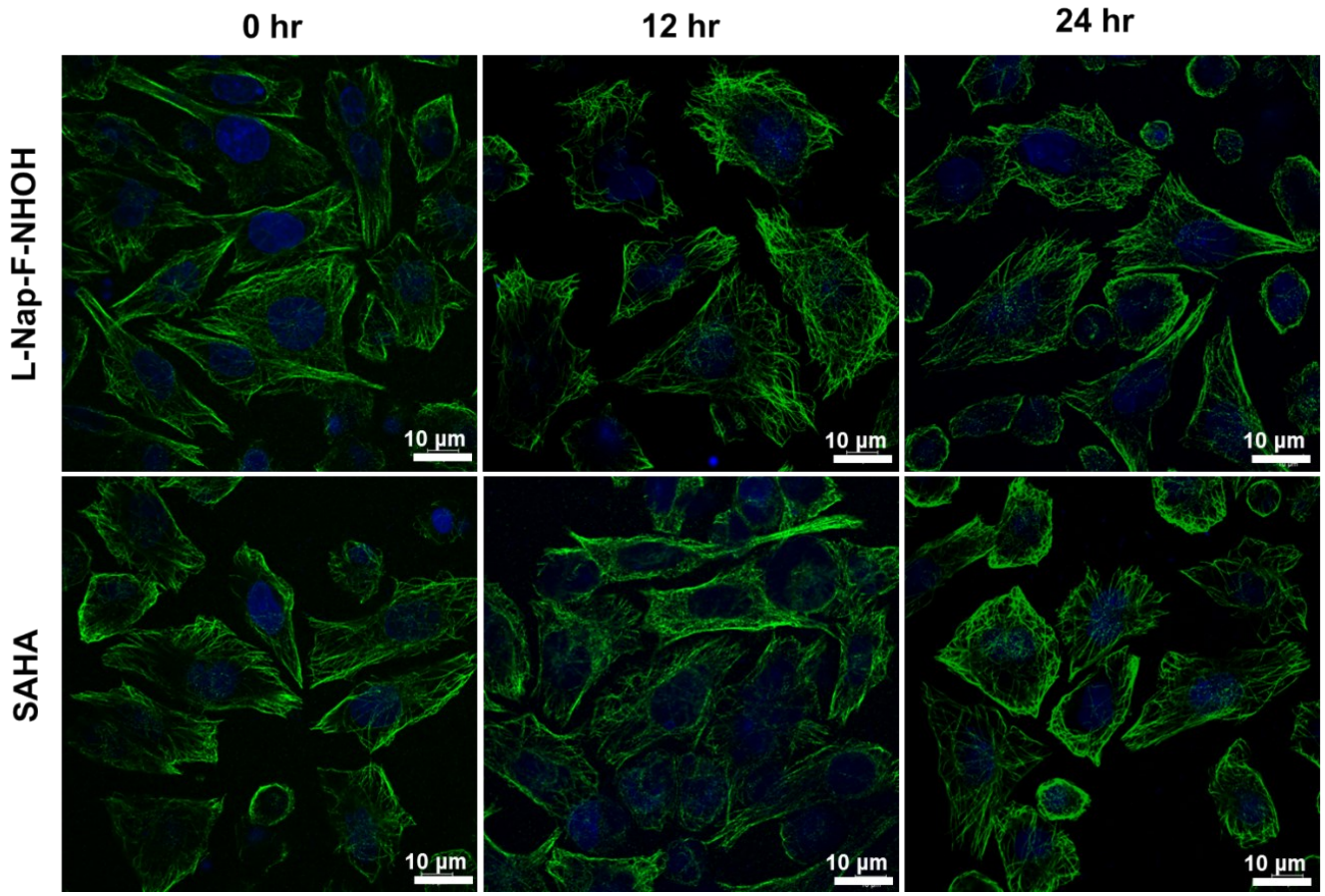


Figure S5: CLSM of microtubule architecture of MDA-MB-231 cell after treatment with compound **4e** (58 nM) for different durations. Distribution of α-tubulin (green) within the cells. Cell nuclei were stained with Hoechst-33342 (blue) (Scale bar = 10 µm).

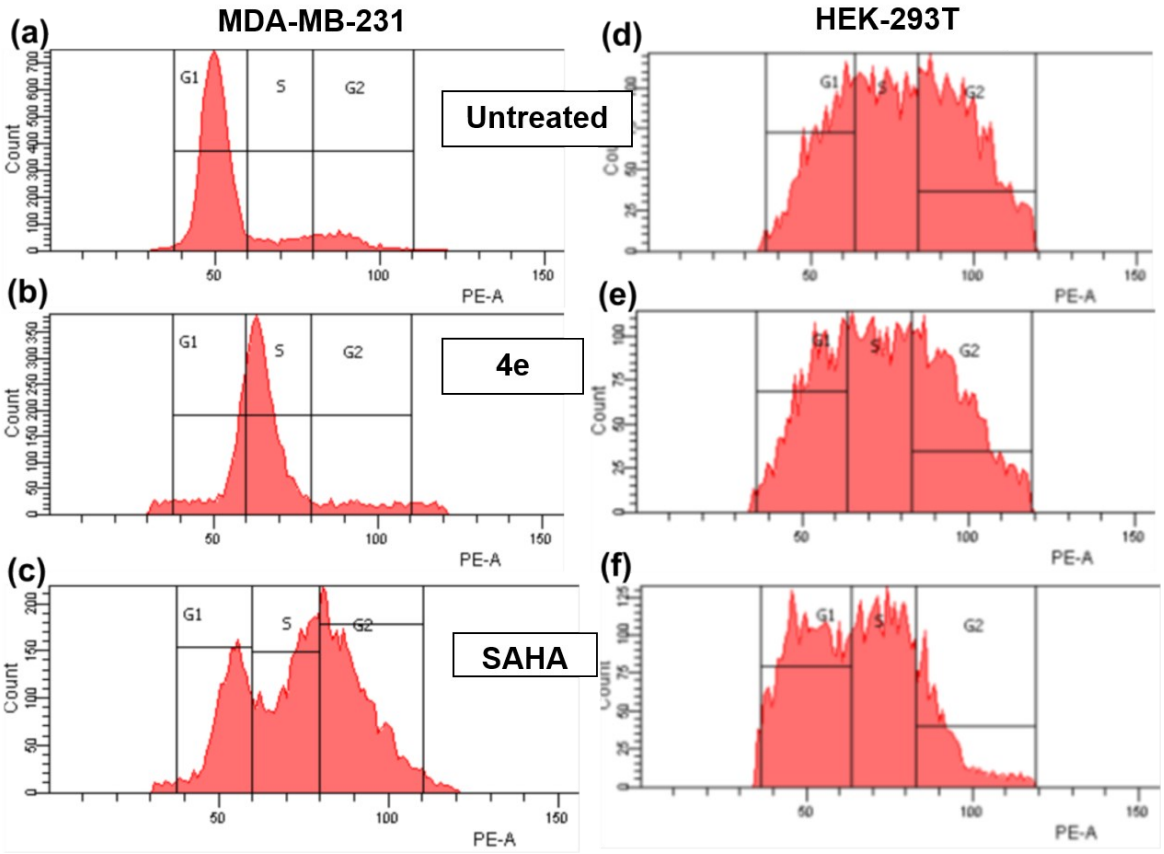


Figure S6: Cell cycle distribution profiles obtained by flow cytometry of MDA-MB-231(left panels, a-c) and HEK-293T (right panels, d-f) after 24 h of treatment with 115 nM of compound **4e** or SAHA. Panels (a) and (d) are for untreated controls; panels (b) and (e) are for cells treated with **4e**; panel (c) and (f) are for cells treated with SAHA.

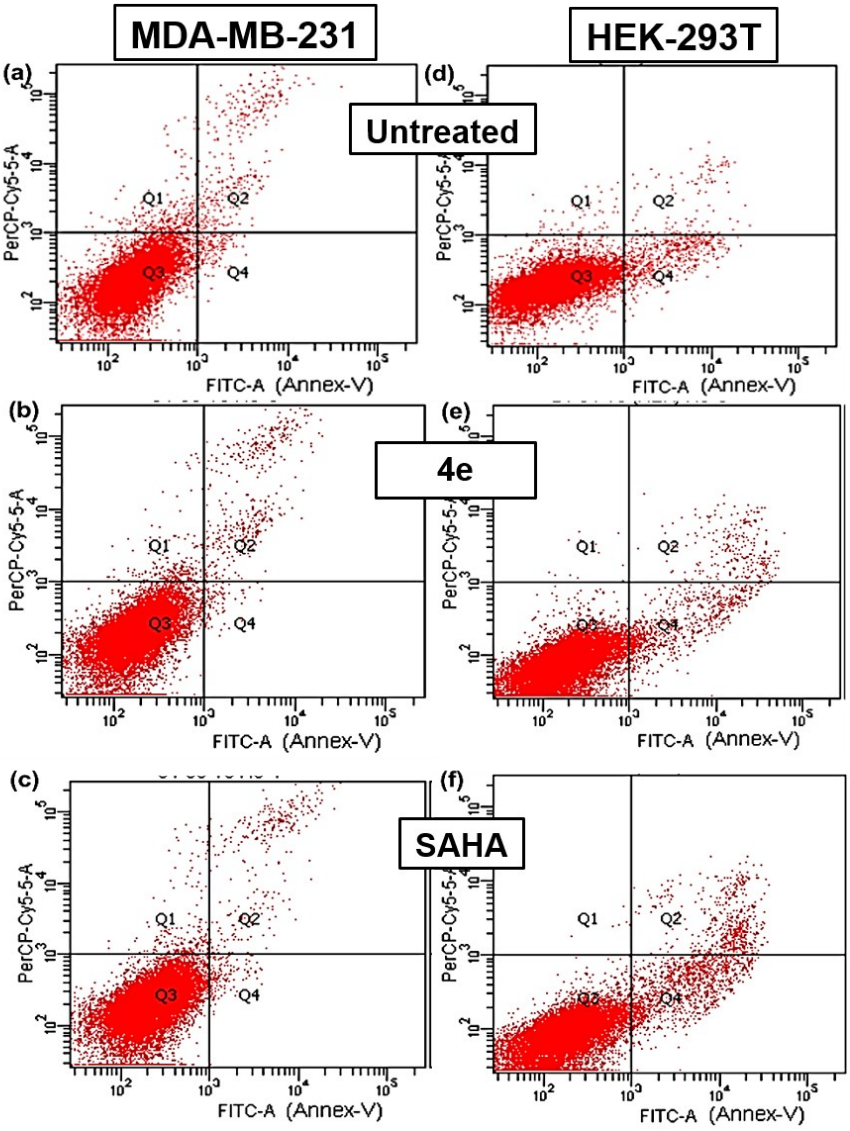


Figure S7: Raw flow cytometry data of AnnexinV/PI stained apoptotic nuclei of untreated (top panels) and after 24 h of treatment by 115 nM of **4e** (middle panels) and SAHA (bottom panels) on MDA-MB-231(left panels) and HEK-293T (right panels).

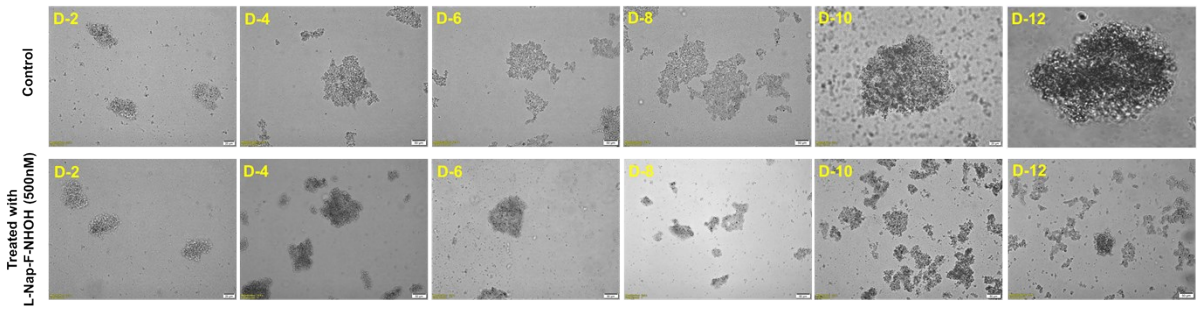


Figure S8: Growth of MCF-7 multicellular tumor spheroids (a) above panel: for control from Day 2 to day 12; (b) Lower Panel: after treatment of compound **4e** at 500 nM concentrations and followed up to day 12. After treatment, the growth of spheroids was monitored by using a CellsDense software and imaging was done with Agilent Fluorescence microscope upto 12 days. (Scale bars represent 50 µm.)

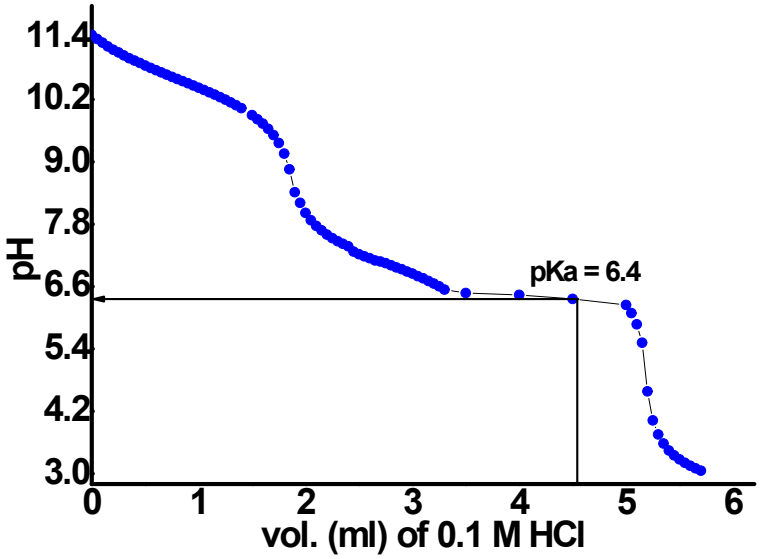


Figure S9: Determination of apparent pK_a value of hydroxamic acid (*hxa*) residue of **4e**.

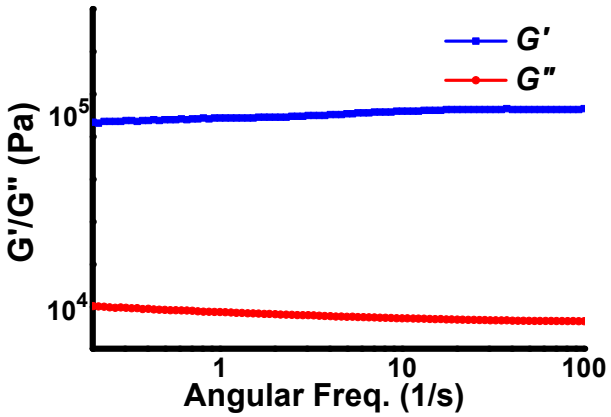


Figure S10: Frequency-sweep rheology on the hydrogel formed by **4e** at 2 mg/mL in water.

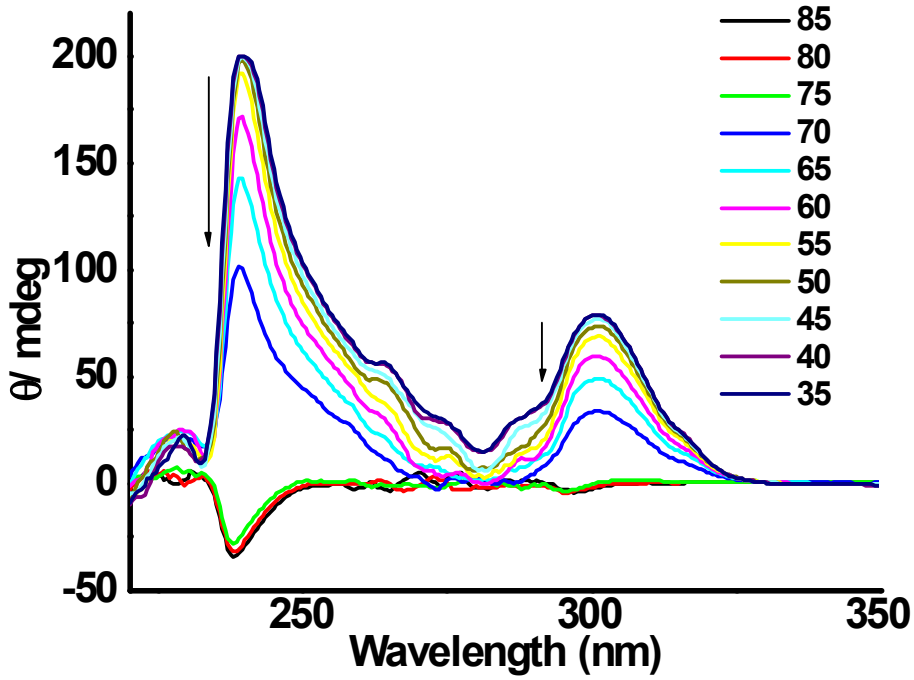


Figure S11: Temperature dependent changes in the circular dichroism (CD) spectrum of **4e** as 1 mg/mL aqueous gel at different temperatures between 35-85 °C. Above 75 °C, the gel had converted to a free flowing sol.

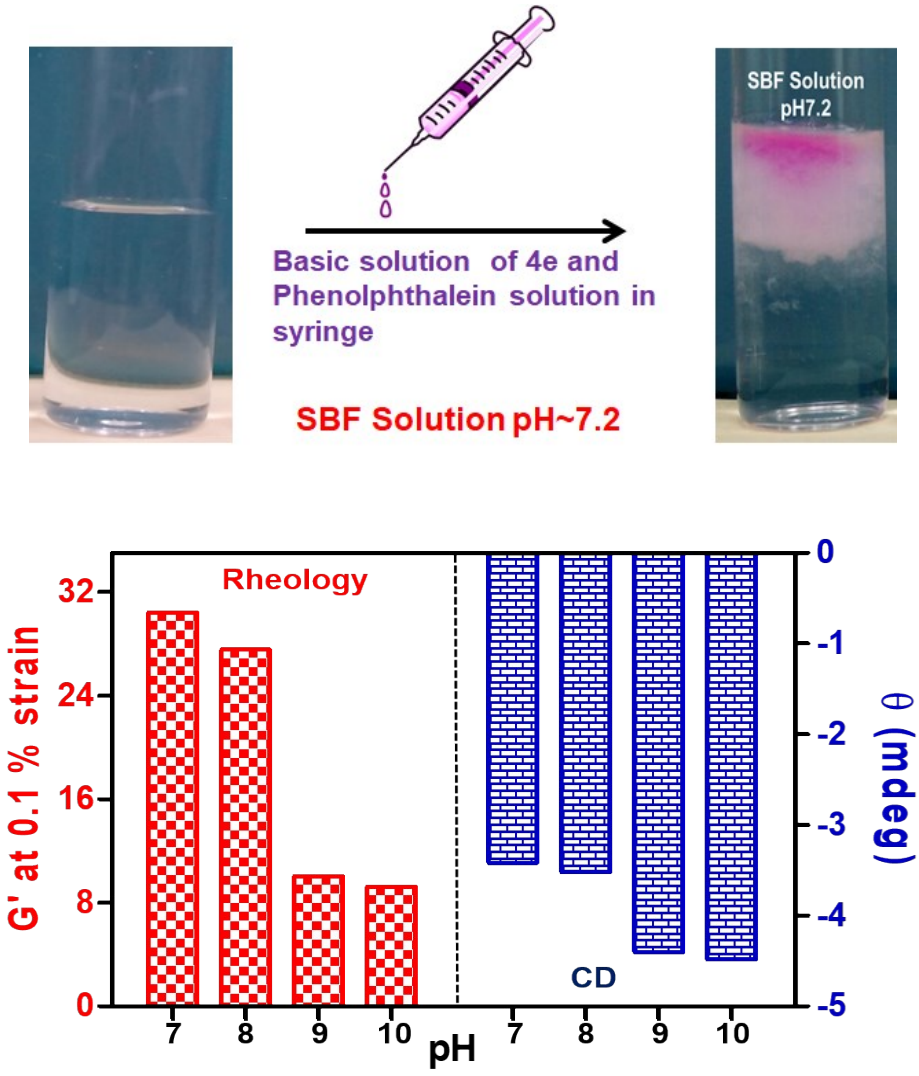


Figure S12: Top panel: pH-induced self-assembly of **4e** (4 mg/mL). A clear solution of **4e** at pH 9.0 was dropped into SBF (pH 7.2). Immediate gelatinous precipitation was noticed on the top of vial. Phenolphthalein was used as the pH indicator. Bottom panel: pH-induced changes in the storage modulus (*G'*) at 0.1% strain (red bars) and ellipticity values at 280 nm (blue bars). Samples prepared in 100 mM carbonate buffer of respective pH. For rheology, the **4e** gels were prepared at 2 mg/ml concentration, for CD the concentration of **4e** was 0.2 mg/ml.

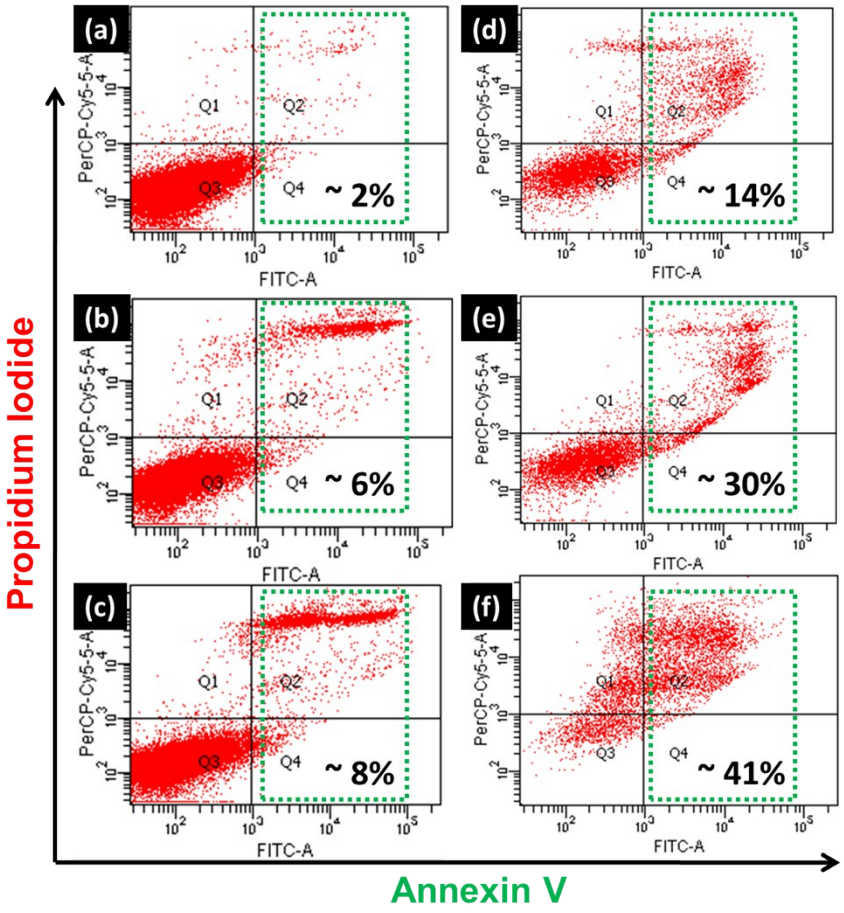


Figure S13: Raw flow cytometry data of FITC-Annexin V and propidium iodide stained apoptotic nuclei of (a) untreated, (b) 6 h, (c) 12 h, (d) 24 h, (e) 36 h and (f) 48 h of treatment by ~11 mM hydrogel of **4e** on MDA-MB-231.

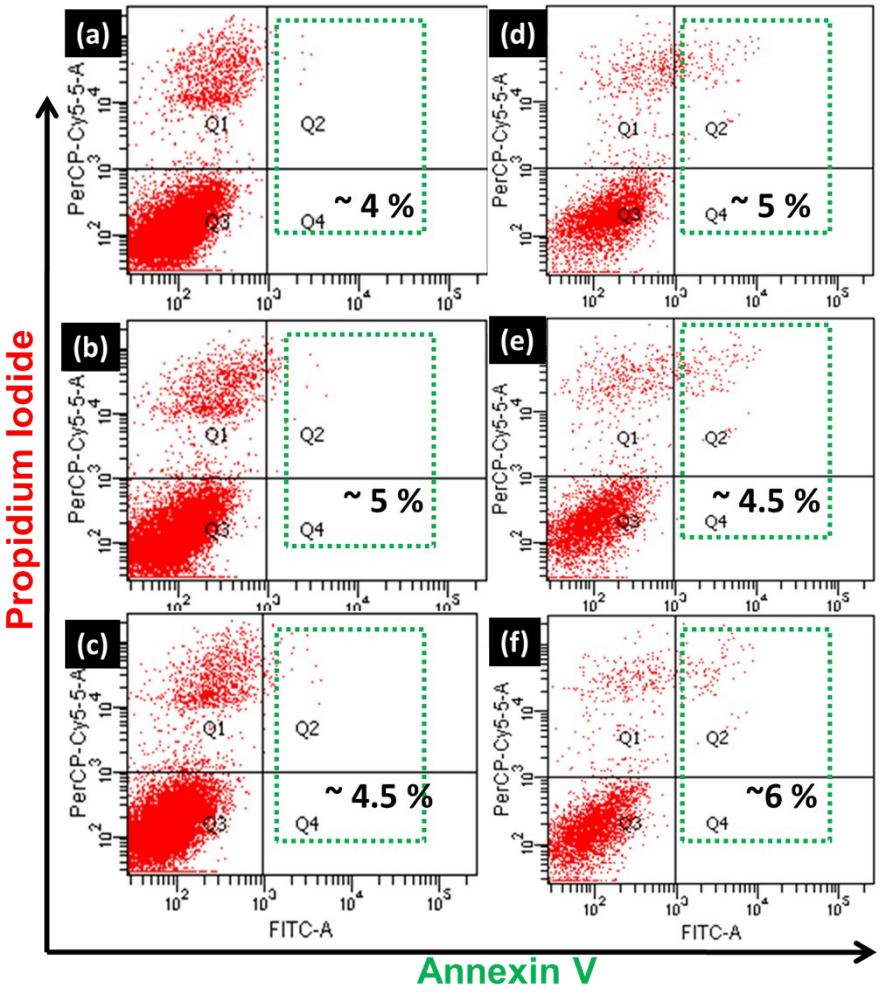


Figure S14: Raw flow cytometry data of FITC-Annexin V and propidium iodide stained apoptotic nuclei of (a) untreated, (b) 6 h, (c) 12 h, (d) 24 h, (e) 36 h and (f) 48 h of treatment by 11 mM hydrogel of **4e** on HEK-293T.

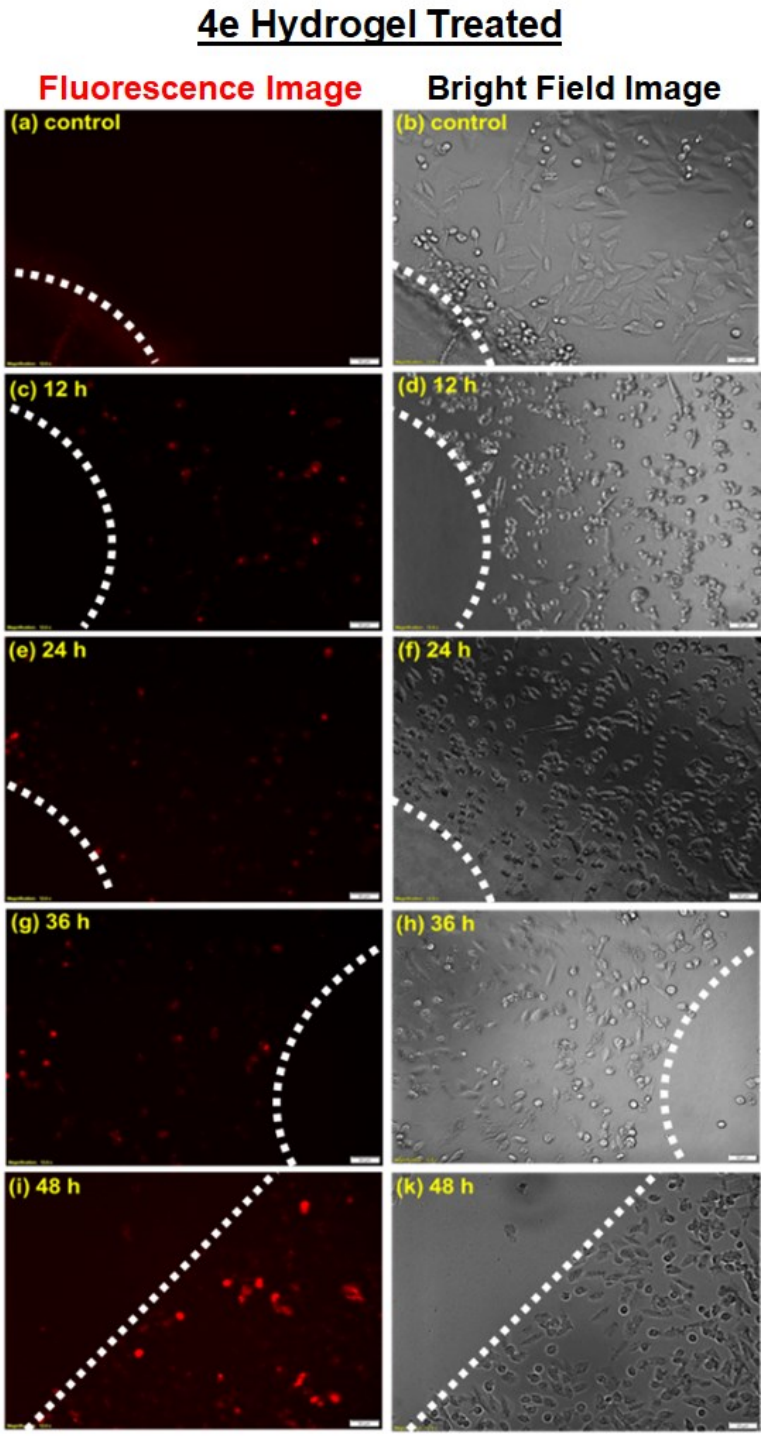


Figure S15: Optical microscopy of **4e** hydrogel treated MDA-MB-231 cells (left panel – red fluorescent images) (right panel - bright field images) at different time point. (a, b) control, (c, d) 12 h, (e, f) 24 h, (g, h) 36 h and (i, j) 48 h, respectively. Scale bar = 50 μm.

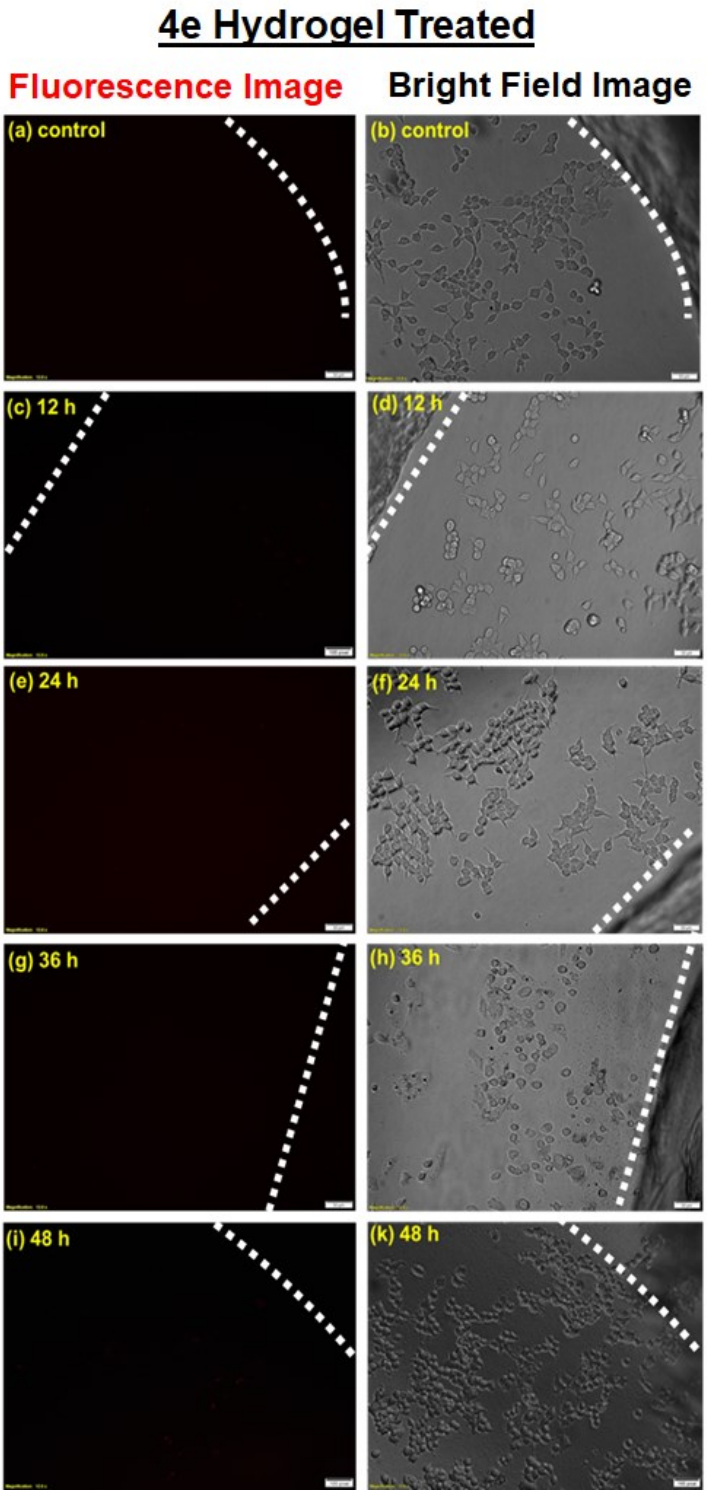


Figure S16: Optical microscopy of **4e** hydrogel on HEK-293T cells (left panel – red fluorescent images) (right panel - bright field images) at different time point. (a, b) control, (c, d) 12 h and (e, f) 24 h, (g, h) 36 h, (i, j) 48 h, respectively. Scale bar = 50 μm.

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2. L. K. Gediya, P. Chopra, P. Purushottamachar, N. Maheshwari, and V. C. O. Njar, *J. Med. Chem.* 2005, 48, **15**, 5047–5051
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