Experimental work

Chapter 5

5. Experimental work

5.1. Materials and methods

All the chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), Alfa Aesar (Massachusetts), S.D. Fine Chemicals (India), CombiBlock (Switzerland), Spectrochem (India), Alfa Aesar (USA), and Avra chemicals (India). All the reagents and solvents were dried using standard methods and distilled prior to use. Chemical reactions were performed under inert atmosphere (N_2) unless otherwise noted. The reactions were monitored by thin-layer chromatography (TLC) on precoated silica gel 60 F_{254} (Merck KGaA) and were visualized under UV light, iodine vapors or by treatment with ninhydrin reagents. Column chromatographic purifications were performed using silica gel 60-120 mesh size (CDH laboratory reagents, India). Proton nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were recorded on Bruker Advance, 500 MHz spectrometers with tetramethylsilane (TMS) as the internal standard. The NMR solvents used were $CDCl_3$ or DMSO-d_6 as indicated. The chemical shifts are reported in ppm and were referenced to the solvent signals, the coupling constants (*J* values) are reported in hertz (Hz). The following abbreviations are used to describe peak splitting patterns where appropriate: d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, br = broad. Coupling constants (*J*) are reported in Hertz (Hz). High-resolution mass spectra (HRMS) were obtained by electrospray ionization, recorded with Agilent1100 LC-Q-TOF and HRMS-6540-UHD machines at Indian Institute of Integrative Medicine (IIIM), Jammu, India, Indian Institute of Technology, Ropar, India, CSIR- Indian Institute of Chemical Technology, Hyderabad and North East Institute of Science & Technology (CSIR–NEIST), Jorhat. The HRMS of the halogenated compounds was measured with reference to major isotope *i.e.* ^{35}Cl, has been carried out and reported. Compounds were named using ChemDraw Professional 15.0 (Perkin Elmer). All

the reagents for biological experiments were purchased from commercial suppliers and used as received unless otherwise indicated. SH-SY5Y cell line was procured from the national center for cell science (NCCS), Pune, India. EZClick™ TUNEL–in situ DNA fragmentation/apoptosis assay kit (K191–001) was purchased from BioVision, U.S.A. Amyloid $\beta_{1\text{-}42}$ fragment (Catalogue# A9810) and hydrogen peroxide 30% solution (CAS No. 107209) were purchased from Sigma Aldrich and Merck Millipore. All the cell-based studies were carried out using a multimode plate reader (Synergy HT, Bio-Tek Instruments, Inc.). PAMPA assay kit was purchased from Bioassay Systems Pvt. Ltd, USA. Scopolamine hydrochloride (CAS No. 55-16-3) and donepezil hydrochloride (CAS No. 120011-70-3) were purchased from Sigma Aldrich.

A. General procedure for the synthesis of compounds 2-chloro-N-phenyl substituted acetamide (2a-2q, and 5a-5b)

To a solution of substituted aniline (**1a-1q**) or 5-amino-indole or 6-aminoquinoline (1.0 equiv.) in dichloromethane (CH_2Cl_2) (15 mL) at 0 °C, potassium carbonate (K_2CO_3) (2.0 equiv.) was added. The reaction mixture was stirred at 0 °C for 15 min. To this stirring solution, chloroacetylchloride (1.0 equiv.) was added dropwise at 0°C and the resultant was allowed to stir for 2 h at room temperature. The progress of the reaction was monitored by TLC using EtOAc:Hexane (1:1). After completion of the reaction, the residue was partitioned between water (20 mL) dichloromethane (3 X 20 mL). The organic solvent phase was concentrated under vacuum to afford the desired compounds **2a**-**2q**, **5a** and **5b**.

2-chloro-N-(phenyl)acetamide (2a). White powder, (0.9g, 66% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.25 (bs, 1H), 7.56 (d, J = 8.0 Hz, 2H), 7.38 (t, J = 7.5 Hz, 2H), 7.19 (t, J = 7.0 Hz, 1H), 4.21 (bs, 2H).

2-chloro-N-(2-methylphenyl)acetamide (2b). White powder, 0.87g, 68% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.65 (bs, 1H), 7.38 (d, J = 7.5 Hz, 1H), 7.23 (d, J = 7.5 Hz, 1H), 7.19-7.17 (m, 1H), 7.13 (dd, J_1 = 7.5 Hz, J_2 = 1.0 Hz, 1H), 4.30 (bs, 2H), 2.20 (s, 3H).

2-chloro-N-(3-methylphenyl)acetamide (2c). White powder, 0.84g, 62% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.21 (bs, 1H), 7.42 (s, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.20 (t, J = 8.0 Hz, 1H), 6.90 (d, J = 7.5 Hz, 1H), 4.23 (bs, 2H), 2.28 (s, 3H).

2-chloro-N-(4-methylphenyl)acetamide (2d). Brown powder, 0.89g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.18 (bs, 1H), 7.47 (d, J = 8.5 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 4.22 (s, 2H), 2.25 (s, 3H).

2-chloro-N-(2-methoxyphenyl)acetamide (2e). White powder, 0.85g, 64% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.27 (bs, 1H), 7.21-7.28 (m, 2H), 7.12 (d, J = 8.0 Hz, 1H), 6.68-6.66 (m, 1H), 4.24 (bs, 2H), 3.73 (s, 3H).

2-chloro-N-(3-methoxyphenyl)acetamide (2f). Black powder, 0.88g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.30 (s, 1H), 7.28 (s, 1H), 7.28-7.21 (m, 1H), 7.13 (t, J = 8.0 Hz, 1H), 6.68- 6.64 (m, 1H), 4.24 (bs, 2H), 3.73 (s, 3H).

2-chloro-N-(4-methoxyphenyl)acetamide (2g). Brown powder, 0.87g, 76% yield. ^{1}H NMR (DMSO-d_6 500 MHz): δ 10.18 (bs, 1H), 7.50 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H), 6.90 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H), 4.21 (s, 2H), 3.72 (s, 3H).

2-chloro-N-(2-chlorophenyl)acetamide (2h). White powder, 0.86g, 71% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.85 (bs, 1H), 7.73 (dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 1H), 7.51 (dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 1H), 7.35 (t, J = 6.5 Hz, 1H), 7.24 (t, J = 6.5 Hz, 1H), 4.37 (s, 2H).

2-chloro-N-(3-chlorophenyl)acetamide (2i). White powder, 0.84g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.65 (bs, 1H), 8.06 (bs, 1H), 7.81-7.80 (m, 1H), 7.55 (dd, J_1 = 4.0 Hz, J_2 = 1.0 Hz, 2H), 4.29 (s, 2H).

2-chloro-N-(4-chlorophenyl)acetamide (2j). White powder, 0.89g, 74% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.39 (bs, 1H), 7.62-7.59 (m, 2H), 7.16 (t, J = 9.0 Hz, 2H), 4.26 (s, 2H).

2-chloro-N-(2-(trifluoromethyl)phenyl)acetamide (2k). White powder, 0.79g, 61% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.92 (bs, 1H), 7.75 (d, J = 8.0 Hz, 1H), 7.70 (t, J = 7.5 Hz, 1H), 7.53-7.47 (m, 2H), 4.32 (s, 2H).

2-chloro-N-(3-(trifluoromethyl) phenyl)acetamide (2l). Brown powder, 0.79g, 61% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.64 (s, 1H), 8.07 (s, 1H), 7.78 (d, J = 8.5 Hz, 1H), 7.57 (t, J = 8.0 Hz, 1H), 7.43 (d, J = 8.0 Hz, 1H), 4.29 (s, 2H).

2-chloro-N-(4-(trifluoromethyl) phenyl)acetamide (2m). White powder, 0.82g, 64% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.24 (bs, 1H), 7.50 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H), 7.33 (dd, J_1 = 7.0 Hz, J_2 = 2.0 Hz, 2H), 4.19 (s, 2H).

2-chloro-N-(3-cyanophenyl)acetamide (2n). Brown powder, 0.81g, 60% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.04 (bs, 1H), 7.79-7.81 (m, 1H), 7.55 (t, J = 7.0 Hz, 2H), 4.27 (bs, 2H).

2-chloro-N-(4-cyanophenyl)acetamide (2o). White powder, 0.88g, 67% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.67 (bs, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 8.0 Hz, 2H), 4.30 (s, 2H).

2-chloro-N-(4-fluorophenyl)acetamide (2p). White powder, 0.86g, 65% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.77 (d, J_1 = 8.5 Hz, 2H), 7.69 (d, J_1 = 8.5 Hz, 2H), 4.27 (bs, 2H).

2-chloro-N-(4-bromophenyl)acetamide (2q). White powder, 0.88g, 67% yield.^{1} H NMR (DMSO-d_6, 500 MHz): δ 7.68-7.76 (m, 1H), 7.82 (bs, 2H), 7.50 (d, J = 8.0 Hz, 1H), 4.30 (bs, 2H).

2-chloro-N-(1H-indol-5-yl)acetamide (5a). Black powder, 0.93g, 78% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.03 (bs, 1H), 10.09 (bs, 1H), 7.86 (bs, 1H), 7.31-7.34 (m, 2H), 7.20 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 6.39 (t, J = 4.5 Hz, 1H), 4.23 (s, 2H).

2-chloro-N-(quinolin-6-yl)acetamide (5b). White powder, 0.91g, 75% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.65 (bs, 1H), 8.80 (dd, J_1 = 4.0 Hz, J_2 = 1.5 Hz, 1H), 8.36 (s, 1H), 8.32 (d, J = 8.0 Hz, 1H), 8.00 (d, J = 5.0 Hz, 1H), 7.80 (dd, J_1 = 9.0 Hz, J_2 = 2.5 Hz, 1H), 7.50 (dd, J_1= 8.0 Hz, J_2= 4.0 Hz, 1H), 4.34 (s, 2H).

B. General procedure for synthesis of compounds 2-amino-*N*-phenyl substituted acetamide (3a-3q, 6a and 6b)

To a solution of substituted phenyl acetamide, **2a-2q** or **5a** or **5b** (0.25g, 1.0 equiv.) excess of liquid ammonia (NH_3) (10mL) was added, and the reaction was heated at 60°C for 6 h. The reaction mixture was allowed to cool to room temperature. The residue was partitioned between water and 30 mL EtOAc. The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo to obtain compounds 3a-3q, 6a, and 6b as a solid/oily product with 50-60% yield. The residue was purified by flash chromatography using DCM:MeOH (9.5:0.5) as eluents to afford the aforementioned compounds.

2-amino-N-(phenyl)acetamide (3a). White solid powder, 0.26g, 55% yield.^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.59 (d, J = 7.5 Hz, 2H), 7.29 (d, J = 7.0 Hz, 2H), 7.03 (t, J = 6.5 Hz, 1H), 3.24 (bs, 2H), 3.15 (s, 2H).

2-amino-N-(o-tolyl)acetamide (3b). White oily product, 0.3g, 60% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.75 (bs, 1H), 7.39-7.44 (m, 2H), 7.10 (t, J = 7.5 Hz, 1H), 6.82 (d, J = 7.0 Hz, 1H), 3.23 (s, 2H), 2.24 (s, 3H).

2-amino-N-(m-tolyl)acetamide (3c). White oily product, 0.28g, 57% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.76 (bs, 1H), 7.45-7.40 (m, 2H), 7.12 (t, J = 8.0 Hz, 1H), 6.85 (d, J = 7.0 Hz, 1H), 3.23 (s, 2H), 2.26 (s, 3H).

2-amino-N-(p-tolyl)acetamide (3d). White oily product, 0.28g, 59% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.38 (bs, 1H), 7.48 (d, J = 8.5 Hz, 2H), 7.27 (d, J = 2.5 Hz, 1H), 7.12 (d, J = 8.0 Hz, 2H), 4.25 (s, 2H), 2.24 (s, 3H).

2-amino-N-(2-methoxyphenyl)acetamide (3e). Oily product, 0.26g, 54% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.83 (d, J = 7.0 Hz, 1H), 7.21-7.15 (m, 2H), 7.02 (d J = 7.0 Hz, 1H), 3.30 (s, 2H), 2.23 (bs, 3H).

2-amino-N-(3-methoxyphenyl)acetamide (3f). Brown oily product, 0.3g, 60% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.77 (bs, 1H), 7.33 (bs, 1H), 7.19-7.16 (m, 2H), 6.61 (d, J = 7.5 Hz, 1H), 3.71 (s, 3H), 3.23 (bs, 2H), 3.16 (s, 2H).

2-amino-N-(4-methoxyphenyl)acetamide (3g). Brown oily product, 0.28g, 58% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.71 (bs, 1H), 7.53 (d, J = 8.0 Hz, 2H), 6.86 (d, J = 8.0 Hz, 2H), 3.71 (s, 3H), 3.22 (bs, 2H), 3.16 (s, 2H).

2-amino-N-(2-chlorophenyl)acetamide (3h). White solid product, 0.26g, 53% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.26 (d, J = 7.5 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.34 (t, J = 7.0 Hz, 1H), 7.13 (t, J = 7.0 Hz, 1H), 3.40 (s, 2H), 3.21 (s, 2H).

2-amino-N-(3-chlorophenyl)acetamide (3i). White solid product, 0.24g, 50% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.18 (bs, 1H), 7.85 (bs, 1H), 7.52 (d, J = 8.0 Hz, 1H), 7.34 (t, J = 8.0 Hz, 1H), 7.11 (d, J = 7.5 Hz, 1H), 3.40 (s, 2H), 3.13 (s, 2H).

2-amino-N-(4-chlorophenyl)acetamide (3j). White solid product, 0.26g, 55% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.71 (bs, 1H), 7.51 (d, J = 7.5 Hz, 2H), 6.84 (d, J = 7.5 Hz, 2H), 3.30 (s, 2H), 3.14 (s, 2H).

2-amino-N-(2-(trifluoromethyl)phenyl)acetamide (3k). Oily product, 0.28g, 58% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.14 (bs, 1H), 7.88 (d, J = 8.0 Hz, 1H), 7.52-7.51 (m, 2H), 3.29 (s, 2H), 3.11 (s, 2H).

2-amino-N-(3-(trifluoromethyl)phenyl)acetamide (3l). Oily product, 0.28g, 56% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.19 (bs, 1H), 7.74 (d, J = 7.5 Hz, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.38 (d, J = 7.5 Hz, 1H), 3.21 (bs, 2H), 3.12 (s, 2H).

2-amino-N-(4-(trifluoromethyl)phenyl)acetamide (3m). Oily product, 0.24g, 51% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.85 (d, J = 8.5 Hz, 2H), 7.66 (d, J = 8.5 Hz, 2H), 4.12 (bs, 2H), 3.30 (bs, 2H).

2-amino-N-(3-cyanophenyl)acetamide (3n). Oily product, 0.28g, 58% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.12 (s, 2H), 7.86 (dd, J_1 = 7.5 Hz, J_2 = 1.5 Hz, 1H), 7.53-7.48 (m, 2H), 3.61 (bs, 2H), 3.29 (bs, 2H).

2-amino-N-(4-cyanophenyl)acetamide (3o). Oily product, 0.30g, 60% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.83 (dd, J_1 = 6.0 Hz, J_2 = 2.5 Hz, 2H), 7.65 (dd, J_1 = 6.0 Hz, J_2 = 2.5 Hz, 2H), 3.44 (bs, 2H), 3.29 (bs, 2H).

2-amino-N-(4-fluorophenyl)acetamide (3p). White solid product, 0.26g, 54% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.65-7.63 (m, 2H), 7.13 (t, J = 9.0 Hz, 2H), 3.62 (bs, 2H), 3.27 (s, 2H).

2-amino-N-(4-bromophenyl)acetamide (3q). Oily product, 0.26g, 54% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.62 (d, J = 8.5 Hz, 2H), 7.47 (d, J = 8.5 Hz, 2H), 4.02 (bs, 2H), 2.98 (bs, 2H).

2-amino-N-(1H-indol-5-yl)acetamide (6a). Oily product, 0.28g, 56% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.98 (bs, 1H), 9.68 (bs, 1H), 7.89 (bs, 1H), 7.30-7.22 (m, 3H), 6.36 (bs, 1H), 3.24 (s, 2H), 3.16 (s, 2H).

2-amino-N-(quinolin-6-yl)acetamide (6b). White solid product, 0.26g, 53% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.77 (bs, 1H), 8.40 (bs, 1H), 8.27 (d, J = 7.5 Hz, 1H), 7.96 (d, J = 8.5 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.47 (bs, 1H), 4.13 (bs, 2H), 3.16 (s, 2H).

C. General procedure for the synthesis of target compounds 4a-4q, 7a, and 7b

Into a stirring solution of trans-ferulic acid (FA) (0.3g, 1.54 mmol) in anhydrous dichloromethane (10 mL), N-hydroxybenzotriazole (HOBt) (0.52g, 3.86 mmol), 1-[3-(dimethyamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDCI.HCl) (0.35g, 2.31 mmol) and DIPEA (0.49g, 3.86 mmol) were added. The reaction was stirred at room temperature for 15 min. Thereafter, a 2-amino-*N*-phenyl substituted acetamide (1.54 mmol) was added, and the reaction mixture was allowed to stir overnight at room temperature. After completion of the reaction, it was quenched by addition of water and extracted with CH_2Cl_2 (3×25 mL). The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated under vacuum. The residue was subjected to silica gel chromatography or crystallization to afford the aforementioned compounds.

(E)-N-(2-((phenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4a). White solid powder, 0.13g, 65% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.58 (d, J = 7.5 Hz, 2H), 7.35 (d, J = 15.5 Hz, 1H), 7.30 (t, J = 8.0 Hz, 2H), 7.15 (bs, 1H), 7.05-7.01 (m, 2H), 6.80 (d, J = 8.0 Hz, 1H), 6.58 (d, J = 16.0 Hz, 1H), 4.00 (bs, 2H), 3.80 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.20, 166.40, 148.66, 148.25, 140.10, 139.16, 129.24, 126.84, 123.80, 122.16, 119.64, 119.54, 118.91, 116.03, 111.33, 56.03, 43.19. ESI-HRMS $[M + H]^+$ Found 327.1340, calculated 327.1345 for $C_{18}H_{18}N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-(o-tolylamino)ethyl)acrylamide (4b). White solid powder, 0.134g, 67% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.48 (bs, 1H), 9.38 (bs, 1H), 8.31 (t, J = 5.5 Hz 1H), 7.44-7.36 (m, 2H), 7.21 (d, J = 7.0 Hz, 1H), 7.16 (t, J = 9.0 Hz, 1H), 7.08 (d, J = 7.0 Hz, 1H), 7.03 (dd, J_1= 6.5 Hz, J_2= 1.5 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.60 (d, J = 15.5 Hz, 1H), 4.06 (s, 2H), 3.81 (s, 3H), 2.20 (bs, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 168.02, 164.84, 149.53, 148.36, 144.95, 136.37, 133.33, 129.65, 126.23, 123.22, 119.87, 116.06, 115.98, 111.59, 56.14, 43.98, 20.90. ESI-HRMS [M + H]$^+$ Found 341.1508, calculated 341.1500 for $C_{19}H_{20}N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-(m-tolylamino)ethyl)acrylamide (4c). White solid powder, 0.122g, 61% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.37 (bs, 1H), 8.30 (bs, 1H), 7.42 (d, J = 7.5 Hz, 1H), 7.37 (d, J = 16.0 Hz, 1H), 7.20 (d, J = 7.0 Hz, 1H), 7.16 (bs, 2H), 7.08 (t, J = 7.0 Hz, 1H), 7.02 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.60 (d, J = 16.0 Hz, 1H), 4.05 (bs, 2H), 3.81 (s, 3H), 2.20 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 168.37, 166.41, 148.84, 148.29, 139.99, 136.53, 131.94, 130.77, 126.81, 126.45, 125.61, 125.12, 122.09, 119.04, 116.13, 111.37, 56.01, 43.23, 18.38. ESI-HRMS [M + H]$^+$ Found 341.1501 calculated 341.1505 for $C_{19}H_{20}N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-(p-tolylamino)ethyl)acrylamide (4d). White solid powder, 0.138g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.48 (bs, 1H), 9.39 (bs, 1H), 8.32 (bs, 1H), 7.43-7.36 (m, 2H), 7.21-7.16 (m, 3H), 7.09-7.01 (m, 2H), 6.80 (d, J = 7.5 Hz, 1H), 6.60 (d, J = 15.5 Hz, 1H), 4.06 (bs, 2H), 3.81 (s, 3H), 2.20 (bs, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ 168.02, 166.34, 148.83, 148.29, 139.96, 136.81, 132.63, 129.56, 126.83, 122.08, 119.05, 116.12, 111.34, 56.02, 43.32, 20.89. ESI-HRMS [M + H]$^+$ Found 341.1503, calculated 341.1505 for $C_{19}H_{20}N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N(2((2-methoxyphenyl)amino)2oxoethyl)acrylamide (4e). White solid powder, 0.124g, 62% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.48 (bs, 1H), 9.19 (bs, 1H), 8.37 (bs, 1H), 8.01 (d, J = 7.5 Hz, 1H), 7.38 (d, J = 15.5 Hz, 1H), 7.17 (bs, 1H), 7.07-7.03 (m, 3H), 6.91 (t, J = 7.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.60 (d, J = 16.0 Hz, 1H), 4.05 (bs, 2H), 3.81 (s, 6H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.90, 165.83, 159.51, 148.37, 147.82, 140.08, 139.47, 129.56, 126.34, 121.58, 118.58, 111.39, 110.90,108.59, 104.96, 55.53, 54.94, 42.91. ESI-HRMS $[M + H]^+$ Found 357.1431 calculated 356.1449 for $C_{19}H_{20}N_2O_5$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-((3-methoxyphenyl)amino)-2-oxoethyl)acrylamide (4f). White solid powder, 0.132g, 66% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.03 (bs, 1H), 9.47 (bs, 1H), 8.26 (t, J = 6.0 Hz, 1H), 7.35 (d, J = 16.0 Hz, 1H), 7.30 (t, J = 1.5 Hz, 1H), 7.23-7.19 (m, 1H), 7.15 (d, J = 2.0 Hz, 1H), 7.12 (d, J = 8.5 Hz, 1H), 7.02 (dd, J_1 = 6.5 Hz, J_2 = 2.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.64-6.58 (m, 2H), 4.01 (bs, 2H), 3.72 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.37, 166.31, 159.97, 148.84, 148.29, 140.55, 139.94, 130.03, 126.80, 122.05, 119.04, 116.13, 111.85, 111.35, 109.06, 105.42, 55.99, 55.40, 43.38. ESI-HRMS $[M + H]^+$ Found 357.1457, calculated 357.1450 for $C_{19}H_{20}N_2O_5$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-((4-methoxyphenyl)amino)-2-oxoethyl)acrylamide (4g). White solid powder, 0.124g, 62% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.89 (bs, 1H), 9.46 (bs, 1H), 8.25 (t, J = 6.0 Hz, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 15.5 Hz, 1H), 7.15 (bs, 1H), 7.02 (dd, J_1 = 6.0 Hz, J_2 = 2.0 Hz, 1H), 6.89 (d, J = 8.0 Hz, 2H), 6.80 (d, J = 7.5 Hz, 1H), 6.60 (d, J = 15.5 Hz, 1H), 3.98 (bs, 2H), 3.81 (s, 3H), 3.71 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.86, 165.81, 159.49, 148.35, 147.80, 140.05, 139.44, 129.53, 126.33, 121.56, 118.57, 115.65, 111.37, 110.90, 108.59, 104.96, 55.52, 54.92, 42.89. ESI-HRMS $[M + H]^+$ Found 357.1458, calculated 357.1450 for $C_{19}H_{20}N_2O_5$.

(*E*)-N-(2-((2-chlorophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4h). White solid powder, 0.140g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.23 (bs, 1H), 9.47 (bs, 1H), 8.31 (t, J = 6.0 Hz, 1H), 7.95 (bs, 1H), 7.80 (bs, 1H), 7.46 (d, J = 8.0 Hz, 1H), 7.37-7.33 (m, 2H), 7.16-7.10 (m, 1H), 7.02 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 16.0 Hz, 1H), 4.02 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.77, 165.82, 148.36, 147.81, 139.45, 135.29, 126.32, 123.98, 121.57, 120.87, 120.81, 118.56, 115.65, 115.38, 115.20, 110.90, 55.53, 42.80. ESI-HRMS $[M + H]^+$ Found 361.0955, calculated 361.0947 for $C_{18}H_{17}ClN_2O_4$.

(*E*)-N-(2-((3-chlorophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4i). White solid powder, 0.138g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.20 (s, 1H), 9.46 (bs, 1H), 8.30-8.27 (m, 2H), 7.93 (t, J = 4.0 Hz, 1H), 7.52-7.50 (m, 1H), 7.35 (d, J = 16.0 Hz, 1H), 7.29-7.22 (m, 2H), 7.15 (d, J = 2.0 Hz, 1H), 7.02 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 6.82 (d, J = 8.0 Hz, 1H), 6.60 (d, J = 15.5 Hz, 1H), 4.01 (s , 2H), 3.81 (s, 3H).^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.75, 166.42, 148.86, 148.30, 140.92, 140.06, 131.24, 126.79, 126.34, 122.11, 122.01, 121.94, 118.93, 118.36, 116.14, 111.39, 56.04, 43.42. ESI-HRMS $[M + H]^+$ Found 361.0959, calculated 360.0945 for $C_{18}H_{17}ClN_2O_4$.

(*E*)-N-(2-((4-chlorophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4j). White solid powder, 0.138g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.55 (s, 1H), 8.38 (t, J = 5.5 Hz, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.50 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H), 7.40-7.32 (m, 2H), 7.20-7.16 (m, 2H), 7.03 (dd, J_1 = 8.5 Hz, J_2 = 1.0 Hz, 1H), 6.81 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 15.5 Hz, 1H), 4.08 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.29, 165.89, 148.37, 147.81, 140.46, 139.54, 130.79, 126.30, 125.84, 121.59, 121.54, 121.41, 118.46, 117.85,

115.65, 110.89, 55.52, 42.94. ESI-HRMS $[M + H]^+$ Found 361.0951, calculated 361.0942 for $C_{18}H_{17}ClN_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-((2(trifluoromethyl)phenyl)amino)ethyl) acrylamide (4k). White solid powder, 0.122g, 61% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.40 (bs, 1H), 9.50 (bs, 1H), 8.30 (bs, 1H), 8.11 (d, J = 7.5 Hz, 1H), 7.85-7.80 (m, 1H), 7.56 (s, 1H), 7.40 (d, J = 15.5 Hz, 1H), 7.38 (s, 1H), 7.16 (s, 1H), 7.02 (d, J = 7.5 Hz, 1H), 6.81 (d, J = 6.0 Hz, 1H), 6.61 (d, J = 15.5 Hz, 1H), 4.01 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.91, 165.97, 148.40, 147.82, 139.67, 135.10, 133.07, 129.07, 126.29, 121.62, 118.33, 115.66, 110.94, 55.53, 42.59. ESI-HRMS $[M + H]^+$ Found 395.1178, calculated 395.1172 for $C_{19}H_{17}F_3N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-((3(trifluoromethyl)phenyl)amino)ethyl) acrylamide (4l). White solid powder, 0.122g, 62% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.82 (bs, 1H), 9.44 (bs, 1H), 8.30 (t, J = 5.5 Hz, 1H), 7.89 (bs, 1H), 7.37 (d, J = 15.5 Hz, 1H), 7.28-7.24 (m, 1H), 7.18-7.15 (m, 2H), 7.02 (dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 2H), 6.81 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 15.5 Hz, 1H), 4.08 (s, 2H), 3.82 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.10, 149.14, 142.77, 138.14, 136.68, 133.63, 128.51, 127.90, 127.13, 126.94, 125.10, 122.79, 122.31, 119.59, 117.17, 110.12, 78.03. ESI-HRMS $[M + H]^+$ Found 395.1101, calculated 395.1120 for $C_{19}H_{17}F_3N_2O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2((4(trifluoromethyl)phenyl)amino)ethyl) acrylamide (4m). White solid powder, 0.130g, 65% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.30 (t, J = 6.0 Hz, 1H), 7.89-7.85 (m, 1H), 7.36 (d, J = 15.0 Hz, 1H), 7.26-7.22 (m, 1H), 7.18-7.12 (m, 3H), 7.02 (dd, J_1 = 5.0 Hz, J_2 = 1.5 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 15.0 Hz, 1H), 4.06 (bs, 2H), 3.80 (bs, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 169.07, 166.39, 148.87, 148.47,

148.30, 142.94, 140.07, 126.78, 126.59, 126.57, 125.92, 123.92, 123.84, 123.76, 123.58 122.09, 119.44, 118.92, 116.14, 111.38, 56.00, 43.47. ESI-HRMS $[M + H]^+$ Found 395.1217, calculated 395.1215 for $C_{19}H_{17}F_3N_2O_4$.

(*E*)-N-(2-((3-cyanophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4n). White solid powder, 0.124g, 62% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.33 (t, J = 5.5 Hz, 1H), 8.06 (bs, 1H), 7.81 (d, J = 8.0 Hz, 1H), 7.55-7.49 (m, 2H), 7.35 (d, J = 16.0 Hz, 1H), 7.15 (bs, 1H), 7.02 (d, J = 7.5 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 16.0 Hz, 1H), 4.02 (bs, 1H), 3.80 (bs, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.95, 166.47, 148.69, 148.23,140.22, 139.96, 130.80, 127.34, 126.78, 124.10, 122.18, 119.16, 118.74,116.02,112.02, 111.32, 56.01, 43.24, 29.43. ESI-HRMS $[M + H]^+$ Found 352.1294, calculated 352.1297 for $C_{19}H_{17}N_3O_4$.

(*E*)-N-(2-((4-cyanophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4o). White solid powder, 0.130g, 65% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.82 (bs, 1H), 9.44 (bs, 1H), 8.29 (t, J = 5.5 Hz 1H), 7.88 (bs, 1H), 7.37 (d, J = 15.5 Hz, 1H), 7.28-7.24 (m, 1H), 7.18-7.15 (m, 3H), 7.02 (d, J = 1.5 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 15.5 Hz, 1H), 4.07 (bs, 2H), 3.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.86, 165.81, 159.49, 148.35, 147.80, 140.05, 139.44, 129.53, 126.33, 121.56, 118.57, 115.65, 111.37, 110.37, 108.59, 104.96, 55.52, 54.92, 42.89. ESI-HRMS (ESI) $[M + H]^+$ Found 378.1457, calculated 378.1461 for $C_{20}H_{17}N_3O_4$.

(*E*)-N-(2-((4-fluorophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4p). White solid powder, 0.120g, 60% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.60 (dd, J_1 = 7.5 Hz, J_2 = 2.5Hz, 2H), 7.34 (d, J = 15.5 Hz, 1H), 7.16-7.13 (m, 3H), 7.02 (dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 15.5 Hz, 1H), 3.99 (bs, 2H), 3.80 (bs, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.14, 166.36, 148.66, 148.23, 140.10, 135.56, 126.81, 122.16, 121.35,

121.29, 118.88, 116.01, 115.89, 115.71, 111.31, 56.01, 43.09. ESI-HRMS $[M + H]^+$ Found 345.1243, calculated 345.1251 for $C_{18}H_{17}FN_2O_4$.

(*E*)-N-(2-((4-bromophenyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (4q). White solid powder, 0.136g, 68% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.18 (s, 1H), 9.46 (s, 1H), 8.29 (t, J = 5.5 Hz, 1H), 7.58 (d, J = 9.0 Hz, 2H), 7.49 (d, J = 9.0 Hz, 2H), 7.35 (d, J = 16.0 Hz, 1H), 7.15 (bs, 1H), 7.02 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 6.59 (d, J = 16.0 Hz, 1H), 4.0 (bs, 2H), 3.18 (bs, 3H).^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.56, 166.33, 148.84, 148.28, 139.99, 138.75, 132.05, 126.79, 122.49, 121.49, 118.98, 116.13, 115.26, 111.36, 56.00, 43.40. ESI-HRMS $[M + H]^+$ Found 407.0420, calculated 407.0426 for $C_{18}H_{17}BrN_2O_4$.

(*E*)-N-(2-((1H-indol-5-yl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (7a). Brown solid powder, 0.140g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.0 (s, 1H), 9.82 (bs, 1H), 9.47 (bs 1H), 8.25 (t, J = 5.5 Hz, 1H), 7.85 (bs, 1H), 7.37-7.29 (m, 3H), 7.20 (dd, J_1 = 9.0 Hz, J_2 = 2.0 Hz, 1H), 7.16 (d, J = 2.0 Hz, 1H), 7.02 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H), 6.80 (d, J = 8.5 Hz, 1H), 6.62 (d, J = 15.5 Hz, 1H), 6.37 (bs, 1H), 4.02 (d, J = 6.0 Hz, 2H), 3.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 169.20, 166.48, 148.85, 148.30, 139.99, 134.18, 133.82, 128.58, 126.82, 126.53, 126.32, 126.06, 125.85, 123.28, 122.10, 119.10, 116.14, 111.34, 56.20, 43.36. ESI-HRMS $[M + H]^+$ Found 366.1451, calculated 366.1446 for $C_{20}H_{19}N_3O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-oxo-2-(quinolin-6-yl-amino)ethyl)acrylamide (7b). Brown solid powder, 0.138g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.41 (bs, 1H), 9.50 (bs, 1H), 8.78 (bs, 1H), 8.35 (bs, 2H), 8.27 (d, J = 7.5 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 9.0 Hz, 1H), 7.47 (s, 1H), 7.37 (d, J = 16.0 Hz, 1H), 7.16 (bs, 1H), 7.02 (d, J = 7.5 Hz, 1H), 6.80 (d, J = 6.5 Hz, 1H), 6.63 (d, J = 15.5 Hz, 1H), 4.08 (s, 2H), 3.81(s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.12,165.76, 148.34, 147.81, 139.34, 132.69, 130.82, 127.42, 126.37, 125.87,

123.69, 121.55, 118.73, 115.66, 114.78, 111.15, 110.90, 110.68, 111.04, 55.53, 42.86. ESI-HRMS $[M + H]^+$ Found 377.1509, calculated 377.1504 for $C_{21}H_{19}N_3O_4$.

D. General procedure for the synthesis of substituted phenylpiperazine compounds 9a-9g

A mixture of substituted aniline (1.0 equiv.), bis(2-chloroethyl) amine hydrochloride (1.0 equiv.), and diethylene glycol monomethyl ether (5 mL) was refluxed at 150°C for 8–10 h under nitrogen atmosphere, cooled to RT. The mixture was dissolved in MeOH (4 mL) followed by addition of Et_2O (100 mL). The resultant precipitate was filtered off and washed with Et_2O to provide HCl salt of **9a**-**9g**, which was used in the next step without purification.

1-phenylpiperazine (9a). Brown liquid, 0.48g, 68% yield).

1-(3-methoxyphenyl)piperazine (9b). Black semi-solid, 0.37g, 69% yield.

1-(4-fluorophenyl)piperazine (9c). Brown semi-solid, 0.41g, 67% yield.

1-(3-nitrophenyl)piperazine (9d). Yellow solid powder, 0.51g, 68% yield.

1-(4-methoxyphenyl)piperazine (9e). Brown solid powder, 0.42g, 66% yield.

1-(4-chlorophenyl)piperazine (9f). Brown semi-solid, 0.39g, 66%.

1-(p-tolyl)piperazine (9g). White solid powder, 0.49 g, 70%.

E. General procedure for the synthesis of compounds 10a-g

To a solution of ferulic acid (0.3 g, 1.54 mmol) and N-substituted-piperazine (1.2 mmol) in tetrahydrofuran (30 mL), N-hydroxybenzotriazole (HOBt) (0.2 g, 1.54 mmol) and 1-[3-(dimethyamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDCI) (0.6 g, 3.86 mmol) and DIPEA (0.49g, 3.86 mmol) were added. The mixture was stirred at RT under nitrogen atmosphere for 10–12 h. After completion of the reaction, the solution was washed successively with 5% HCl, saturated $NaHCO_3$ and brine. then dried over anhydrous Na_2SO_4, and concentrated under vacuum.

The crude material was purified by column chromatography over silica gel (hexane/EtOAc, 6:4) to give the final compound.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-phenylpiperazin-1-yl)prop-2-en-1-one (10a). White solid product, 0.160g, 80% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.44 (bs, 1H), 7.44 (d, J = 15.0 Hz, 1H), 7.34 (s, 1H), 7.23 (t, J = 8.0 Hz, 2H), 7.12-7.09 (m, 2H), 6.97 (d, J = 8.0 Hz, 2H), 6.83-6.77 (m, 2H), 3.83 (s, 5H), 3.71 (bs, 2H), 3.16 (s, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.37, 151.32, 148.97, 148.32, 142.87, 129.46, 127.16, 123.08, 119.79, 116.34, 115.90, 114.87, 111.66, 56.27, 49.27, 48.95, 45.27, 41.99. ESI-HRMS [M + H]$^+$ Found 339.1708, calculated 339.1702 for $C_{20}H_{22}N_2O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(3-methoxyphenyl)piperazin-1-yl)prop-2-en-1-one (10b). White solid product, 0.164g, 82% yield**.** ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.47 (s, 1H), 7.44 (d, J = 15.0 Hz, 1H), 7.35 (s, 1H), 7.13-7.10 (m, 2H), 6.94 (d, J = 8.5 Hz, 2H), 6.84 (d, J = 5.0 Hz, 2H), 6.77 (d, J = 6.5 Hz, 1H), 3.69 (bs, 5H), 3.68 (bs, 5H), 3.02 (s, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.33, 153.79, 148.95, 148.32, 145.67, 142.85, 127.16, 123.08, 118.51, 115.89, 114.88, 114.77, 111.63, 56.26, 55.64, 51.11, 50.44, 45.44, 42.10. ESI-HRMS [M + H]$^+$ Found 369.1816, calculated 369.1818 for $C_{21}H_{24}N_2O_4$.

((*E*)-1-(4-(4-fluorophenyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (10c). Pale yellow solid product, 0.162g, 81% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.44 (bs, 1H), 7.43 (d, J = 15.0 Hz, 1H), 7.34 (bs, 1H), 7.12-7.05 (m, 4H), 7.01 (bs, 2H), 6.78 (d, J = 15.0 Hz, 1H) 3.83 (s, 5H), 3.71 (bs, 2H), 3.09 (bs, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 165.36, 157.71, 155.83, 148.97, 148.32, 148.23, 148.22, 142.90, 127.15, 123.08, 118.25, 118.19, 115.90, 115.72, 114.84, 111.66, 56.27, 50.37, 49.74, 45.28, 42.00. ESI-HRMS [M + H]$^+$ Found 357.1611, calculated 357.1605 for $C_{20}H_{21}FN_2O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(3-nitrophenyl)piperazin-1-yl)prop-2-en-1-one (10d). Yellow solid product, 0.166g, 83% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.45 (bs, 1H), 7.44 (d J = 15.0 Hz, 1H), 7.34 (s, 1H), 7.12–7.06 (m, 4H), 7.01-6.99 (m, 2H), 6.77 (d, J = 8.0 Hz, 1H), 3.83 (s, 5H), 3.80 (bs, 2H), 3.09 (s, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 164.93, 157.28, 155.40, 148.53, 147.88, 147.80, 142.51, 126.70, 122.69, 117.83, 117.77, 115.48, 115.45, 115.30, 114.37, 111.16, 55.81, 49.93, 49.31, 44.85, 41.55. ESI-HRMS [M + H]$^+$ Found 384.1561, calculated 384.1556 for $C_{20}H_{21}FN_2O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(4-methoxyphenyl)piperazin-1-yl)prop-2-en-1-one (10e). White solid product, 0.170g, 85% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.47 (s, 1H), 7.43 (d, J = 15.0 Hz, 1H), 7.35 (bs, 1H), 7.13-7.10 (m, 2H), 6.94 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 9.0 Hz, 2H), 6.78 (d, J = 8.0 Hz, 1H), 3.68 (s, 5H), 3.35 (s, 5H), 3.02 (s, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 164.89, 153.34, 148.50, 147.87, 145.22, 142.45, 126.71, 122.67, 118.08, 115.43, 114.40, 114.31, 111.12, 55.80, 55.19, 50.75, 47.56, 42.32, 41.89. ESI-HRMS [M + H]$^+$ Found 369.1819, calculated 369.1812 for $C_{21}H_{24}N_2O_4$.

(*E*)-1-(4-(4-chlorophenyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (10f). White solid product, 0.166g, 83% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.44 (bs, 1H), 7.43 (d, J = 15.5 Hz, 1H), 7.32 (bs, 1H), 7.10-6.98 (m, 6H), 6.78 (d, J = 8.5 Hz, 1H), 3.83 (bs, 7H), 3.08 (bs, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 170.16, 162.48, 160.60, 153.72, 153.06, 152.93, 147.70, 131.89, 127.79, 123.00, 122.94, 120.65, 120.45, 119.55, 116.43, 116.35, 61.01, 55.13, 54.54, 50.06, 46.75. ESI-HRMS [M + H]$^+$ Found 373.1502, calculated 373.1509 for $C_{20}H_{21}ClN_2O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(p-tolyl)piperazin-1-yl)prop-2-en-1-one (10g). White solid product, 0.160g, 80% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): ^{1}H NMR (DMSO-d_6,

500 MHz): δ 9.44 (bs, 1H), 7.44 (d, J = 15.5 Hz, 1H), 7.32 (bs, 1H), 7.21 (bs, 1H), 7.10-7.07 (m, 2H), 6.91 (d, J = 8.5 Hz, 1H), 6.83-6.77 (m, 3H), 3.83 (bs, 6H), 3.68 (bs, 6H), 3.01 (bs, 4H). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 170.14, 158.56, 153.71, 153.07, 150.38, 147.65, 131.91, 127.78, 123.27, 120.66, 119.59, 119.50, 116.39, 61.00, 60.38, 55.84, 55.20, 50.27, 46.93. ESI-ESI-HRMS $[M + H]^+$ Found 353.1254, calculated 353.1260 for $C_{21}H_{24}N_2O_3$.

F. General procedure for the synthesis of intermediates 12e-12g, 12i, 12n-12o

To a stirring solution of substituted benzyl halide (1.0 equiv, 1 mmol) in ethanol (30 mL), K_2CO_3 (3.0 equiv, 3.0 mmol), the appropriate anhydrous piperazine (4.0 equiv) were added, followed by the general procedure [180]. After refluxing for 4-6 h, the ethanol was evaporated under reduced pressure, and the residue was diluted with EtOAc (20 ml) and washed with ice-cold water. The aqueous layer was extracted with EtOAc (3 × 20 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated to yield the aforementioned compounds **12e-12g, 12i, 12n-12o**, which were used without purification in the next step. The intermediates were characterized with the help of ^{1}H-NMR.

1-(3-methoxybenzyl)piperazine (12e). White solid powder, (0.22g, 73% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): 7.25-7.22 (m, 1H, Ar-H), 6.86-6.88 (m, 2H), 6.82 (dd, J_1 = 6.0 Hz, J_1 = 2.0 Hz, 1H), 3.80 (s, 3H), 3.56 (bs, 2H), 3.19 (t, J = 5.0 Hz, 4H), 2.72 (bs, 4H).

1-(4-methoxybenzyl)piperazine (12f). White solid powder, (0.21g, 70% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.89 (bs, 1H), 7.37 (d, J = 8.5 Hz, 2H), 6.99 (d, J = 8.5 Hz, 2H), 4.06 (bs, 2H), 3.77 (s, 4H, merged with DMSO-d_6), 3.07 (bs, 4H, merged with DMSO-d_6), 2.64 (s, 3H).

1-(2-chlorobenzyl)piperazine (12g). White solid powder, (0.27g, 69% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.48-7.46 (m, 1H), 7.36-7.34 (m, 1H), 7.26-7.17 (m, 2H), 3.62 (bs, 2H), 3.15 (bs, 2H), 2.96 (t, J = 9.5 Hz, 4H), 2.55 (bs, 4H).

1-(4-chlorobenzyl)piperazine (12i). White solid powder, (0.29g, 74% yield). ^{1}H NMR (DMSO-d_6, 500 MHz): 7.35 (d, J = 8.5 Hz, 2H), 7.30 (t, J = 7.0 Hz, 2H), 3.43-3.41 (m, 2H, merged with DMSO-d_6), 3.41 (bs, 2H), 2.69 (bs, 2H), 2.27 (bs, 4H).

3-(piperazin-1-ylmethyl)benzonitrile (12n). White solid powder, (0.23g, 78% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): 8.20 (bs, 1H), 8.10 (d, J = 7.0 Hz, 1H), 7.67 (d, J = 7.5 Hz, 1H), 7.50-7.47 (m, 1H), 3.58 (s, 2H), 3.09-2.94 (m, 4H), 2.48 (bs, 4H).

4-(piperazin-1-ylmethyl)benzonitrile (12o). White solid powder, (0.21g, 70% yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.35 (bs, 1H), 8.19-8.16 (m, 2H), 7.71-7.28 (m, 2H), 3.13 (d, J = 11.5 Hz, 2H), 2.98-2.96 (m, 4H), 2.59 (bs, 4H).

G. General procedure for the synthesis of target compounds 13a-13p

Into a stirring solution of FA (0.2 g, 1.0 equiv.) in dry THF (20 mL), EDCI. HCl, 0.29g, 1.5 equiv.), HOBt (0.37 g, 2.5 equiv.), and (DIPEA ,0.33 g, 2.5 equiv.) were added. The desired amount of either commercially (**12a-12d**, **12f**, **12h**, **12m 12k-12l**) or synthesized derivative (**12e-12g**, **12i**, **12n-12o**) (0.8 equiv.) was added to the reaction flask and the reaction mixture was allowed to stir stirred under nitrogen atmosphere at RT for 12 h. The progress of reaction was monitored using Hexane/EtOAc as mobile phase. Upon completion of the reaction, THF was removed under reduced pressure, followed by the addition of saturated $NaHCO_3$ solution. The reaction mixture was extracted with EtOAc (3 × 30 mL), and the combined organic layer was dried over Na_2SO_4, and evaporated under reduced pressure to obtain the crude product. Further purification was carried by column chromatography over silica gel with the help of hexane/EtOAc (1:1).

(*E*)-1-(4-benzylpiperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13a). White solid powder, 0.145g, 75% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.44 (s, 1H), 7.40 (d, J = 15.2 Hz, 1H), 7.34-7.25 (m, 6H), 7.08-7.02 (m, 2H), 6.76 (d, J = 8.0 Hz, 1H), 3.81 (bs, 3H),

3.69 (bs, 2H), 3.54-3.50 (m, 4H), 2.39-2.34 (m, 4H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 165.25, 148.91, 148.30, 142.70, 138.28, 129.40, 128.66, 127.49, 127.17, 122.97, 115.89, 114.95, 111.63, 62.33, 56.25, 53.72, 52.82, 45.46, 42.11. ESI-HRMS [M + H]+ Found 353.1864, calculated 353.1865 for $C_{21}H_{24}N_2O_3$.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(2-methylbenzyl)piperazin-1-yl)prop-2-en-1-one (13b.HCl). White solid powder, 0.200g, 69% yield. 1H NMR ($CDCl_3$, 500 MHz): δ 7.60 (d, J = 15.5 Hz, 1H), 7.24 (d, J = 7.5 Hz, 1H), 7.19-7.14 (m, 3H), 7.08 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 6.98 (d, J = 2.0 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 6.70 (d, J = 15.5 Hz, 1H), 3.91 (s, 3H), 3.72-3.62 (m, 4H), 3.48 (s, 2H), 2.48 (t, J = 5.0 Hz, 4H), 2.37 (s, 3H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 171.17, 165.67, 147.33, 146.71, 142.87, 137.59, 135.83, 130.39, 129.91, 127.89, 127.31, 125.56, 121.85, 114.75, 114.59, 109.92, 60.75, 60.40, 55.99, 21.04, 19.24. ESI-HRMS [M + H]+ Found 367.2031, calculated 367.2022 for $C_{22}H_{26}N_2O_3$.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(3-methylbenzyl)piperazin-1-yl)prop-2-en-1-one (13c.HCl). White solid powder, 0.200g, 69% yield. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.62 (d, J = 15.5 Hz, 1H), 7.24 (t, J = 6.0 Hz, 1H), 7.16-7.10 (m, 4H), 7.00 (d, J = 5.0 Hz, 1H), 6.93 (d, J = 7.5 Hz, 1H), 6.73 (d, J = 15.5 Hz, 1H), 3.94 (s, 3H), 3.76-3.67 (m, 4H), 3.52 (s, 2H), 2.50 (d, J = 6.0 Hz, 4H), 2.37 (s, 3H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 165.51, 149.25, 148.25, 143.73, 138.51, 132.47, 130.62, 129.82, 129.17, 128.98, 126.89, 123.18, 115.95, 114.07, 111.86, 59.56, 56.29, 21.40. ESI-HRMS $[M + H]^+$ Found 367.2013, calculated 367.2022 for $C_{22}H_{26}N_2O_3$. **(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(4-methylbenzyl)piperazin-1-yl)prop-2-en-1-one (13d).** White solid powder, 0.200g, 68% yield. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.62 (d, J = 15.5 Hz, 1H), 7.22 (d, J = 8.0 Hz, 2H), 7.16 (d, J = 8.00 Hz, 2H), 7.10 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 7.00 (d, J = 6.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 1H), 6.72 (d, J = 15.5 Hz, 1H), 3.94 (s, 3H), 3.76-3.67 (m,

4H), 3.53 (s, 2H), 2.50 (t, J = 5.0 Hz, 4H), 2.36 (s, 3H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.64, 147.31, 146.69, 142.88, 137.00, 129.19, 129.04, 127.89, 121.86, 114.74, 114.56, 109.88, 62.60, 55.99, 31.93, 29.70, 22.69, 21.11, 14.12. ESI-HRMS $[M + H]^+$ Found 367.2018, calculated 367.2022 for $C_{22}H_{26}N_2O_3$.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(3-methoxybenzyl)piperazin-1-yl)prop-2-en-1-one (13e). White solid powder, 0.210g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.51 (bs, 1H), 7.49-7.42 (m, 3H), 7.30 (d, J = 7.5 Hz, 1H), 7.12-6.99 (m, 4H), 6.78 (t, J = 8.5 Hz, 1H), 3.82-3.70 (m, 6H), 3.53 (bs, 4H), 3.12 (bs, 2H), 2.97 (bs, 4H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 168.32, 166.15, 159.97, 140.54, 140.18, 139.58, 130.05, 129.09, 128.55, 128.16, 127.17, 113.27, 111.83, 110.84, 109.05, 105.40, 67.27, 58.23, 55.99, 55.40, 51.65. ESI-HRMS $[M + H]^+$ Found 383.1968, calculated 383.1971 for $C_{22}H_{26}N_2O_4$.

(E)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(4-methoxybenzyl)piperazin-1-yl)prop-2-en-1-one (13f). White solid powder, 0.210g, 69% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.61 (d, J = 15.3 Hz, 1H), 7.25 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.2 Hz, 1H), 6.99 (s, 1H), 6.92-6.87 (m, 3H), 6.71 (d, J = 15.3 Hz, 1H), 3.93 (s, 3H), 3.82-3.69 (m, 7H), 3.53 (s, 2H), 2.51 (s, 4H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.67, 159.01, 147.40, 146.75, 142.98, 130.51, 127.82, 121.90, 114.80, 114.44, 113.76, 109.90, 62.16, 55.98, 55.28, 53.13, 52.55, 45.67, 42.01, 29.69. ESI-HRMS $[M + H]^+$ Found 383.1970, calculated 383.1971 for $C_{22}H_{26}N_2O_4$.

(*E*)-1-(4-(2-chlorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13g.HCl). White solid powder, 0.214g, 69% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.62 (d, J = 15.3 Hz, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.39 (dd, J_1 = 7.8 Hz, J_2 = 2.5 Hz, 1H), 7.26-7.21 (m, 2H, merged with $CDCl_3$), 7.12-7.10 (m, 1H), 7.01-7.12 (m, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.73 (d, J = 15.3 Hz, 1H), 3.94 (s, 3H), 3.77 (s, 4H), 3.68 (s, 2H), 2.58 (t, J = 5.0 Hz, 4H).^{13}C NMR ($CDCl_3$,

126 MHz): δ 165.68, 147.33, 146.70, 142.93, 135.32, 134.46, 130.80, 129.59, 128.42, 127.88, 126.67, 121.88, 114.74, 114.53, 109.89, 59.13, 56.00, 29.70. ESI-HRMS $[M + H]^+$ Found 387.1471, calculated 387.1475 for $C_{21}H_{23}ClN_2O_3$.

(*E*)-1-(4-(3-chlorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13h.HCl). White solid powder, 0.214g, 71% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.61 (d, J = 15.3 Hz, 1H), 7.36 (s, 1H), 7.25 (s, 2H), 7.28-7.21 (m, 3H), 7.10 (d, J = 8.0 Hz, 1H), 6.99 (bs, 1H), 6.92 (d, J = 8.2 Hz, 1H), 6.70 (d, J = 15.3 Hz, 1H), 3.92 (s, 3H), 3.76-3.68 (m, 4H), 3.52 (s, 2H), 2.49 (t, J = 5.5 Hz, 4H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 165.71, 147.47, 146.27, 143.07, 139.84, 134.3, 129.62, 129.09, 127.75, 127.52, 127.16, 121.94, 114.82, 114.33, 109.94, 62.24, 55.91. ESI-HRMS $[M + H]^+$ Found 387.1470, calculated 387.1475 for $C_{21}H_{23}ClN_2O_3$.

(*E*)-1-(4-(4-chlorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13i.HCl). White solid powder, 0.214g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.62 (d, J = 15.3 Hz, 1H), 7.32–7.27 (m, 4H), 7.10 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H), 6.99 (d, J = 2.0 Hz, 1H), 6.62 (d, J = 8.0 Hz, 1H), 6.71 (d, J = 15.3 Hz, 1H), 3.92 (s, 3H), 3.75–3.67 (m, 4H), 3.51 (s, 2H), 2.48 (t, J = 5.5 Hz, 4H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.74, 147.41, 146.77, 143.07, 136.15, 133.08, 130.33, 128.51, 127.72, 121.84, 114.87, 114.32, 109.91, 62.0, 55.94. ESI-HRMS $[M + H]^+$ Found 387.1477, calculated 387.1475 for $C_{21}H_{23}ClN_2O_3$.

(*E*)-1-(4-(2-fluorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13j.HCl). White solid powder, 0.216g, 71% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.58 (d, J = 15.0 Hz, 1H), 7.35 (td, J_1 = 7.5 Hz, J_2 = 2.0 Hz, 1H), 7.25-7.22 (m, 1H), 7.10 (td, J_1 = 8.0 Hz, J_2 = 1.0 1H), 7.05-7.00 (m, 2H), 6.96 (d, J = 5.5 Hz, 1H), 6.87 (d, J = 8.0 Hz, 1H), 6.68 (d, J = 15.5 Hz, 1H), 3.86 (s, 3H), 3.75-3.72 (m, 4H), 3.62 (bs, 2H), 2.52 (t, J = 5.0 Hz, 4H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.78, 162.39, 160.44, 147.77, 147.07, 143.18, 131.68, 131.65, 129.19, 129.12,

127.55, 123.97, 123.95, 123.85, 123.73, 121.95, 115.43, 115.25, 115.09, 114.11, 110.08, 67.93, 55.90, 55.09, 55.08, 52.95, 52.44, 25.59. ESI-HRMS $[M + H]^+$ Found 371.1769, calculated 371.1771 for $C_{21}H_{23}FN_2O_3$.

(*E*)-1-(4-(3-fluorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13k.HCl). White solid powder, 0.216g, 73% yield. ^1H NMR ($CDCl_3$, 500 MHz): δ 7.62 (d, J = 15.5 Hz, 1H), 7.32-7.28 (m, 1H), 7.14-7.09 (m, 3H), 7.01-7.00 (m, 2H), 6.92 (d, J = 8.5 Hz, 1H), 6.71 (d, J = 15.5 Hz, 1H), 3.94 (s, 3H), 3.79-3.71 (m, 4H), 3.58 (s, 2H), 2.54 (s, 4H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 168.82, 165.29, 148.91, 148.29, 142.77, 138.05, 129.02, 127.44, 127.15, 123.01, 121.57, 115.88, 114.90, 111.62, 61.84, 56.25, 49.06. ESI-HRMS $[M + H]^+$ Found 371.1776, calculated 371.1771 for $C_{21}H_{23}FN_2O_3$.

(*E*)-1-(4-(4-fluorobenzyl)piperazin-1-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (13l.HCl). White solid powder, 0.210g, 69% yield. ^1H NMR (DMSO-d_6, 500 MHz): δ 7.59 (d, J = 15.5 Hz, 1H), 7.29-7.27 (m, 2H), 7.07 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 7.08-6.97 (m, 3H), 6.90 (d, J = 8.0 Hz, 1H), 6.70 (d, J = 15.0 Hz, 1H), 3.91 (s, 3H), 3.65-3.73 (bs, 4H), 3.49 (s, 2H), 2.46 (t, J = 5.0 Hz, 4H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 165.71, 163.14, 161.18, 147.40, 146.74, 143.04, 130.69, 130.63, 127.80, 121.88, 115.28, 115.11, 114.79, 114.39, 109.92, 62.03, 55.98, 53.18, 52.70, 31.93, 29.70, 27.09, 19.73, 14.12. ESI-HRMS $[M + H]^+$ Found 371.1775, calculated 371.1771 for $C_{21}H_{23}FN_2O_3$.

(*E*)-2-((4-(3-(4-hydroxy-3-methoxyphenyl)acryloyl)piperazin-1-yl)methyl)benzonitrile (13m.HCl). White solid powder, 0.213g, 71% yield. ^1H NMR (DMSO-d_6, 500 MHz): δ 7.69-7.54 (m, 4H), 7.40 (t, J = 8.5 Hz, 1H), 7.11-7.09 (m, 1H), 7.00 (bs, 1H), 6.92 (d, J = 7.75 Hz, 1H), 6.72 (d, J = 15.5 Hz, 1H), 3.93 (s, 3H), 3.75-3.69 (m, 6H), 2.57 (s, 4H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.71, 147.34, 146.72, 143.04, 141.86, 133.15, 132.65, 130.14, 127.81, 127.84, 121.93, 117.73,

114.77, 114.44, 113.18, 109.85, 60.34, 56.07. ESI-HRMS $[M + H]^+$ Found 378.1814, calculated 378.1818 for $C_{22}H_{23}N_3O_3$.

(*E*)-3-((4-(3-(4-hydroxy-3-methoxyphenyl)acryloyl)piperazin1yl)methyl)benzonitrile (13n. HCl). Brown solid powder, 0.213g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.69–7.58 (m, 4H), 7.45 (t, J = 8.5 Hz, 1H), 7.10 (dd, J_1 = 8.0 Hz, J_2 = 2.0 Hz, 1H), 7.00 (d, J = 2.0 Hz, 1H), 6.92 (d, J = 8.15 Hz, 1H), 6.71 (d, J = 15.5 Hz, 1H), 3.94 (s, 3H), 3.76 (s, 4H), 3.57 (s, 2H), 2.49 (t, J = 5.5 Hz, 4H). ^{13}C NMR ($CDCl_3$, 126 MHz): δ 165.72, 147.44, 146.75, 143.15, 139.54, 133.33, 132.33, 131.07, 129.18, 121.87, 118.84, 114.72, 112.54, 109.97, 61.87, 55.90. ESI-HRMS $[M + H]^+$ Found 378.1816, calculated 378.1818 for $C_{22}H_{23}N_3O_3$.

(*E*)-4-((4-(3-(4-hydroxy-3-methoxyphenyl)acryloyl)piperazin-1-yl)methyl)benzonitrile (13o.HCl). White solid powder, 0.195g, 66% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.52 (s, 1H), 8.14 (s, 1H), 7.84 (d, J = 8.0 Hz, 1H), 7.62 (d, J = 8.0 Hz, 1H), 7.50-7.46 (m, 2H), 7.33 (bs, 1H), 7.14-7.06 (m, 2H), 6.80 (d, J = 8.0 Hz, 1H), 4.51-4.25 (m, 4H), 3.83 (m, 3H), 3.61-3.56 (m, 4H), 3.17 (s, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 168.49, 166.18, 149.86, 149.52, 140.18, 139.63, 138.34, 129.15, 129.08, 128.55, 128.13, 127.23, 127.16, 113.26, 110.83, 67.28, 58.23, 55.99, 51.65, 47.87, 43.37. ESI-HRMS $[M + H]^+$ Found 378.1826, calculated 378.1818 for $C_{22}H_{23}N_3O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-1-(4-(3-nitrobenzyl)piperazin-1-yl)prop-2-en-1-one (13p.HCl). Yellow solid powder, 0.251g, 70% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.90 (bs, 1H), 9.43 (bs, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.42-7.30 (m, 4H), 7.08-7.02 (m, 2H), 6.77 (d, J = 8.5 Hz, 1H), 3.82-3.63 (m, 8H), 3.19 (bs, 2H), 2.53 (bs, 3H, merged with DMSO-d_6). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 168.80, 165.36, 148.91, 148.29, 142.78, 137.97, 128.98, 127.52, 127.15,

122.97, 121.61, 115.89, 114.88, 111.60, 61.80, 56.52, 56.25, 55.29, 53.67, 52.94, 18.97. ESI-HRMS $[M + H]^+$ Found 398.1719, calculated 398.1716 for $C_{21}H_{23}ClN_2O_3$.

H. General procedure for the synthesis of 3-((tert-butoxycarbonyl)amino)propanoic acid (15).

Into a stirring solution alanine (0.8 g, 9.09 mmol) in DCM (30 mL), $(Boc)_2O$ (2.37 g, 10.90 mmol) and NaOH (0.29 g, 7.27 mmol) were added at 0°C. The reaction mixture was stirred at room temperature for 12 h and was extracted with DCM (3 × 30 mL), washed with water, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude material was purified by column chromatography over silica gel (hexane/EtOAc, 9.0:1.0) to give compound **5.** White solid powder yield 80%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.26 (s, 1H), 5.12 (s, 1H), 3.41 (d, J = 5.5 Hz, 2H), 2.59-2.54 (m, 2H), 1.47 (s, 9H).

I. General procedure for the synthesis of intermediates 16a-16c

Into a stirring solution of compound **14** (0.20 g, 1 mmol) in DCM (20 mL), substituted amine (0.19 g, 0.8 mmol), HOBt ,(0.37 g, 2.5 mmol), 1-Ethyl-3-EDCI.HCl (0.29 g, 1.5 mmol) and DIPEA (0.33 g, 2.5 mmol) were added at room temperature, and the reaction mixture was stirred for 8-10 h. Upon completion of the reaction, the reaction mixture was partitioned between saturated brine solution DCM (3 × 30 mL). The combined organic layer was dried in a vacuum, and purified by silica gel column chromatography to yield compound (**16a**-**16c**).

tert-butyl (3-oxo-3-(phenylamino)propyl)carbamate (16a). White solid powder, 0.225g, yield 85%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.02 (bs, 1H), 09.97 (s, 1H). 7.88 (d, J = 8.5 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 7.33 (t, J = 6.5 Hz, 1H), 3.66 (t, J = 6.5 Hz, 2H), 3.19 (t, J = 7.0 Hz, 2H), 1.51 (s, 9H).

tert-butyl (3-((3-methoxyphenyl)amino)-3-oxopropyl)carbamate (16b). White solid powder, 0.242g, yield 82%. ^{1}H NMR ($CDCl_3$ 500 MHz): δ 7.81 (q, J_1 = 9.5 Hz, J_2 = 7.0 Hz, 1H), 7.48-7.45 (m, 1H), 7.30 (bs, 1H), 7.22 (t, J = 8.0 Hz, 2H), 7.03 (d, J = 7.5 Hz 1H), 6.68 (dd, J_1 = 6.5 Hz, J_2 = 1.5 Hz, 1H), 3.81 (s, 3H), 3.49 (q, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 2.62 (t, J = 6.5 Hz, 2H), 1.44 (s, 9H).

tert-butyl (3-((4-chlorophenyl)amino)-3-oxopropyl)carbamate (16c). Brown solid powder, 0.230g, yield 81%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 7.98 (d, J = 8.5 Hz, 1H), 7.72 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 9.0 Hz, 1H), 7.54 (t, J = 7.0 Hz, 1H), 7.41 (t, J = 6.5 Hz, 1H), 7.33 (d, J = 8.5 Hz, 1H), 3.20 (t, J = 6.5 Hz, 2H), 3.20 (t, J = 6.0 Hz, 2H), 1.37 (s, 9H).

J. General procedure for the synthesis of intermediates 17a-17c

Into a stirring solution of compound **16a-16c** (0.2 g, 1.0 mmol) in DCM (30 mL), TFA (15 mL) was added slowly at room temperature, and the reaction mixture was stirred for 6 h. The solvent was removed in vacuo, and thus formed salt was washed with ether. The resultant mixture saturated solution of $NaHCO_3$ was added, and it was extracted with DCM (50 × 3 mL). The combined organic layer was dried over Na_2SO_4, filtered, and evaporated in vacuo to provide the compound 1**7a**-1**7c**, which was subjected to the next reaction without further purification.

J. General procedure for the synthesis of target compounds 18a-18c

Into a stirring solution of FA (0.3 g, 1.54 mmol) in dry THF (10 mL), HOBt (0.52 g, 3.86 mmol), EDCI.HCl (0.35 g, 2.31 mmol) and DIPEA (0.49 g, 3.86 mmol) were added. The reaction was stirred at room temperature for 15 min. Finally, substituted amine (1.0 equiv.) was added, and the reaction mixture was allowed to stir overnight at RT. The progress of the reaction was monitored by TLC. After completion of the reaction, the saturated $NaHCO_3$ solution was added slowly into it, and the mixture was extracted with ethyl acetate (3 × 50 mL), and the combined organic layer

was washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was subjected to silica gel chromatography or crystallization to afford the aforementioned target compounds.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(3-oxo-3-(phenylamino)propyl) acrylamide (18a). White solid powder, 0.228g, yield 76%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.10 (s, 1H), 9.43 (s, 1H), 8.06 (t, J = 11.5 Hz, 1H), 7.64 (d, J = 9.0 Hz, 2H), 7.36-7.30 (m, 3H), 7.11 (d, J = 2.0 Hz, 1H), 6.97 (dd, J_1 = 8.0 Hz, J_2 = 1.5 Hz, 1H), 6.78 (d, J = 8.5 Hz, 1H), 6.48 (d, J = 15.5 Hz, 1H), 3.79 (s, 3H), 3.45 (q, J_1 = 12.5 Hz, J_2 = 6.5 Hz, 2H), 2.55 (t, J = 6.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 166.46, 149.45, 148.85, 148.28, 140.07, 137.28, 135.95, 129.95, 128.77, 126.79, 123.74, 122.23, 122.11, 118.93, 116.11, 115.42, 111.32, 56.00, 43.50. ESI-HRMS $[M + H]^+$ Found 341.1497, calculated 341.1501 for $C_{21}H_{23}ClN_2O_3$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(3-((3-methoxyphenyl)amino)-3-oxopropyl)acrylamide (18b). White solid powder, 0.154g, 71% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.66 (bs, 1H), 9.44 (bs, 1H), 8.08 (s, 1H), 7.34-7.30 (m, 2H), 7.20-7.11 (m, 4H), 6.98 (dd, J_1 = 8.0 Hz, J_2 = 2.5 Hz, 1H), 6.78 (d, J = 8.0 Hz, 1H), 6.61 (dd, J_1 = 8.0 Hz, J_2 = 3.0 Hz, 1H), 6.47 (d, J = 15.5 Hz, 1H), 3.83-3.79 (m, 3H), 3.71 (s, 3H), 3.45 (q, J_1 = 12.5 Hz, J_2 = 6.5 Hz, 2H), 2.54 (t, J = 6.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 169.24, 166.18, 148.79, 148.28, 139.80, 136.69, 127.65, 126.86, 123.09, 122.04, 121.36, 119.21, 118.69, 116.12, 112.18, 111.82, 111.31, 56.01, 42.75, 25.64. ESI-HRMS $[M + H]^+$ Found 371.1605, calculated 371.1607 for $C_{20}H_{22}N_2O_5$.

(*E*)-N-(3-((4-chlorophenyl)amino)-3-oxopropyl)-3-(4-hydroxy-3-methoxyphenyl) acrylamide (18c). White solid powder, 0.168g, 74% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.97 (bs, 1H), 9.43 (s, 1H), 8.08 (t, J = 5.5 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.33-7.27 (m, 3H),

7.11-6.97 (m, 3H), , 6.77 (d, J = 8.0 Hz, 1H), 6.47 (d, J = 15.5 Hz, 1H), 3.79 (s, 3H), 3.45 (dd, J_1 = 8.0 Hz, J_2 = 2.5 Hz, 2H), 2.55 (t, J = 6.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 169.98, 166.00, 148.70, 148.26, 139.65, 139.47, 129.13, 126.85, 123.55, 122.04, 119.53, 119.36, 116.07, 111.13, 55.95, 36.89, 35.63. ESI-HRMS $[M + H]^+$ Found 375.1115, calculated 375.1112 for $C_{19}H_{19}ClN_2O_4$.

K. General procedure for the synthesis of intermediate 21a-21c.

Into a stirring solution of Boc-glycine (0.3 g, 2.55 mmol) in THF (20 mL), substituted tryptamine (0.4 g, 2.55 mmol), EDCI.HCl ,0.97 g, 4.59 mmol), HOBt ,0.512 g, 3.82 mmol), and DIPEA (,0.15 g, 2.55 mmol) was added at RT. After the reaction was stirred for 12 h, diluted solution of HCl was added into the reaction mixture, and it was extracted with EtOAc (3 × 50 mL). The combined organic layer was washed with brine and finally purified by silica gel column chromatography (EtOAc/Hexane, 1:1) to get the aforementioned product.

tert-butyl (2-((2-(1H-indol-3-yl)ethyl)amino)-2-oxoethyl)carbamate (21a). Brown solid powder, 0.190g, 71% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.80 (bs, 1H), 7.85 (t, J = 5.5 Hz, 1H), 7.52 (d, J = 8.0 Hz, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.13 (bs, 1H), 7.06 (t, J = 8.0 Hz, 1H), 6.97 (t, J = 7.5 Hz, 1H), 6.91 (t, J = 6.0 Hz, 1H), 3.50 (d, J = 8.5 Hz, 2H), 3.34 (q, J_1 = 7.0 Hz, J_2 = 6.5, 2H), 2.80 (t, J = 6.5 Hz, 2H), 1.38 (s, 9H).

tert-butyl(2-((2-(5-methoxy-1H-indol-3-yl)ethyl)amino)-2-oxoethyl)carbamate (**21b**). Brown solid powder, 0.202g, 73% yield. ^{1}H NMR (CDCl$_3$, 500 MHz): δ 10.65 (bs, 1H), 7.85 (t, J = 5.5 Hz, 1H), 7.21 (d, J = 9.0 Hz, 1H), 7.10 (d, J = 2.0 Hz, 1H), 7.02 (d, J = 2.0 Hz, 1H), 6.92 (t, J = 7.0 Hz, 1H), 6.70 (dd, J_1 = 6.0 Hz, J_2 = 2.5 Hz, 1H), 3.71 (s, 3H), 3.51 (d, J = 6.5 Hz, 2H), 2.77 (t, J = 7.5 Hz, 2H), 1.38 (s, 9H).

tert-butyl (2-((2-(5-chloro-1H-indol-3-yl)ethyl)amino)-2-oxoethyl)carbamate (**21c**). White solid powder, 0.254g, 75% yield. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 11.03 (bs, 1H), 7.86 (t, J = 5.0 Hz, 1H), 7.34 (d, J = 8.5 Hz, 1H), 7.23 (bs, 1H), 7.05 (dd, J_1 = 8.5 Hz, J_2 = 2.0 Hz, 1H), 6.93 (t, J = 6.0 Hz, 1H), 3.50 (d, J = 6.0 Hz, 2H), 3.31 (t, J = 6.5 Hz, 2H), 2.78 (t, J = 7.5 Hz, 2H), 1.38 (s, 9H).

L. General procedure for the synthesis of intermediate 22a-22c.

Into a stirring solution of compound **21a**-**21c** (0.3g, 1.0 mmol) in methanol (20 mL), ether HCl (3 mL) was added slowly at room temperature, and the reaction mixture was stirred for 12 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the Et_2O (10 mL) was added to provide HCl salt. The HCl salt was further converted to a free amine by treatment with Na_2CO_3 solution and extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in vacuo to provide the pure free amine product **22a**-**22c**, which were subjected to the next reaction without further purification.

(*E*)-N-(2-((2-(1H-indol-3-yl)ethyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (23a). Brown solid powder, 0.152g, 71% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.78 (bs, 1H), 9.44 (bs, 1H), 8.14 (t, J = 6.0 Hz, 1H), 8.01 (t, J = 6.0 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.35-7.31 (m, 2H), 7.14 (t, J = 4.5 Hz, 2H), 7.06-6.95 (m, 3H), 6.79 (d, J = 8.0 Hz, 1H), 6.56 (d, J = 15.5 Hz, 1H), 3.80-3.78 (m, 5H), 3.34 (s, 2H, merge with DMSO-d_6), 2.82 (t, J = 7.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 169.24, 166.18, 148.79, 148.29, 139.80, 136.69, 127.65, 126.86, 123.09, 122.04, 121.36, 119.21, 118.69, 116.12, 112.18, 111.82, 111.31, 56.01, 42.75, 25.64. ESI-HRMS $[M + H]^+$ Found 394.1773, calculated 394.1767 for $C_{22}H_{23}N_3O_4$.

(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-(2-((2-(5-methoxy-1H-indol-3-yl)ethyl)amino)-2-oxoethyl)acrylamide (23b). White solid powder, 0.158g, 66% yield.^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.65 (bs, 1H), 9.44 (bs, 1H), 8.15 (t, J = 6.0 Hz 1H), 8.03 (t, J = 6.0 Hz, 1H), 7.35 (d, J = 16.0 Hz, 1H), 7.22 (d, J = 9.0 Hz, 1H), 7.13 (dd, J_1 = 6.5 Hz, J_2 = 2.0 Hz, 2H), 7.03-7.01 (m, 2H), 6.80 (d, J = 8.0 Hz, 1H), 6.71 (dd, J_1 = 6.5 Hz, J_2 = 2.0 Hz, 1H), 6.58 (d, J = 16.0 Hz, 1H), 3.81 (s, 5H), 3.76 (s, 3H), 3.36 (t, J = 6.0 Hz, 2H), 2.80 (t, J = 7.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 169.23, 166.13, 153.45, 148.79, 148.28, 139.76, 131.83, 127.97, 126.86, 123.80, 122.01, 119.25, 116.14, 112.47, 111.94, 111.51, 111.33, 100.53, 55.99, 55.81, 42.76, 25.71. ESI-HRMS $[M + H]^+$ Found 424.1874, calculated 424.1872 for $C_{23}H_{25}N_3O_5$.

(*E*)-N-(2-((2-(5-chloro-1H-indol-3-yl)ethyl)amino)-2-oxoethyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide (23c). White solid powder, 0167g, 69% yield. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 11.03 (s, 1H), 9.52 (s, 1H), 8.18 (t, J = 6.0 Hz, 1H), 8.04 (t, J = 6.0 Hz, 1H), 7.59 (d, J = 2.0 Hz, 1H), 7.37-7.34 (m, 2H), 7.25 (d, J = 2.0 Hz, 1H), 7.05 (dd, J_1 = 6.0 Hz, J_2 = 2.0 Hz, 2H), 6.80 (d, J = 8.0 Hz, 1H), 6.57 (d, J = 16.0 Hz, 1H), 3.81 (s, 5H), 3.34-3.32 (m, 4H), 2.80 (t, J = 7.5 Hz, 2H). ^{13}C NMR (DMSO-d_6, 126 MHz): δ 148.28, 139.75, 135.14, 128.83, 126.86, 125.14, 123.45, 122.01, 121.29, 119.23, 117.99, 116.13, 113.36, 112.18, 111.32, 65.39, 55.99, 42.75, 25.59, 25.45, 21.53, 15.64. ESI-HRMS $[M + H]^+$ Found 428.1376, calculated 428.1377 for $C_{21}H_{23}ClN_2O_3$.

5.2. Biological evaluation

Determination of IC_{50} values

Human acetylcholinesterase (*h*AChE), from human erythrocytes AChE (CAS No. 9000-81-1, Sigma Aldrich), butyryl cholinesterase from *equine* serum *eq*BChE (CAS NO. 9001-08-5, Sigma Aldrich), 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent, DTNB CAS No.69-78-3),

acetylthiocholine iodide (ATCI, CAS No. 1866-15-5) and butyrylthiocholine iodide (BTCI, CAS No. 1866-16-6) were purchased from Sigma Aldrich. The determinations of AChE inhibitory capacity of the compounds were tested by Ellman assay with slight modification. Ferulic acid (Sigma Aldrich, CAS No. 1261170-81-3) and DPZ hydrochloride (Sigma Aldrich, CAS No. 120011-70-3) were used as reference compounds. All the experiments were conducted in 50 mM Tris-HCl buffer at pH 8. Briefly, 50 μL of AChE (0.022 U mL^{-1}) and 10 μL of the test or standard compound were incubated in 96-well plates at room temperature for 30 min. Additional, 30 μL of the substrate viz. ATCI (1.5 mM) was added, and the solution was incubated for an additional 30 min at room temperature. Finally, 160 μL of DTNB (0.15 mM) was added to it, and after 30 sec, the absorbance was measured at 415 nm wavelength using a 96 well microplate reader (Synergy^{TM} HT, Bio-Tek Instruments, Inc., and Epoch 2 microplate reader, Bio-Tek Instruments, Inc.). Each assay was performed in triplicate, and each experiment was repeated at least two-three times independently. The blank contained all components except the enzyme. The inhibition percent was calculated using the following expression: [(Ac−Ai)/Ac] X 100, where Ai and Ac are the absorbances obtained for AChE in the presence and absence of inhibitors. The *in-vitro* BChE assay was performed using the same procedure as mentioned above. Briefly, 50 μL of BChE (0.06 U mL^{-1}) and 10 μL of the test or standard compound were incubated in 96-well plates at room temperature for 30 min. Further, 30 μL of the substrate viz. BTCI (15 mM) was added, and the solution was incubated for an additional 30 min at room temperature. Finally, 160 μL of DTNB (1.5 mM) was added to it, and after 30 sec the absorbance was measured at 415 nm wavelength using a 96-well microplate reader. Each assay was performed in triplicate, and each experiment was repeated at least two-three times independently. The inhibition percent was calculated by the

following expression: [(Ac-Ai)/Ac] X 100, where Ai and Ac are the absorbances obtained for BChE in the presence and absence of inhibitors.

Kinetic characterization of AChE and BChE inhibition

To study the ChE inhibitory mechanism, reciprocal plots of 1/[V] versus 1/[S] were constructed using six different concentrations of the substrate acetylthiocholine iodide (ATCI) or five concentrations of butyryl thiocholine iodide (BTCI) (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM for *h*AChE; 5, 10, 15, 20, and 25 mM for equine BChE) by using the Ellman method. Briefly, 10 μL compound was pre-incubated with *h*AChE (50 μL of 0.022 U/mL) or *eq* BChE (50 μL of 0.06 U/mL) and DTNB (160 μL of 0.15 mM for AChE; 160 µL of 1.5 mM for *eq*BChE), at 37 ºC for 30 min, followed by the addition of 30 μL of the substrate at different concentrations. The kinetic characterization of the hydrolysis of ATCI or BTCI catalyzed by AChE or *eq*BChE was done spectrometrically using a 96-well microplate reader (Synergy™ HT, Bio-Tek Instruments, Inc. and Epoch 2 microplate reader, Bio-Tek Instruments, Inc.) at 415 nm.

Propidium iodide displacement assay

A solution of *h*AChE at a concentration of 5.0 U/mL in 0.1 mM Tris buffer, pH 8.0, was used. Aliquots of the compounds (1:1) to get the final concentrations of 5, 10, 20, and 50 μM were added, and the solutions were kept at room temperature for 6 h at 25 °C. Finally, the samples were incubated for 20 min with propidium iodide at a final concentration of 20 μM, and the fluorescence was measured in a fluorescence microplate reader (Synergy™ HT, Bio-Tek Instruments, Inc.). The wavelength of excitation and emission were 535 and 595 nm, respectively.

DPPH radical-scavenging potency

The DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on the reduction of DPPH. DPPH gets reduced in the presence of an antioxidant molecule, giving rise to a yellow-colored diphenyl picrylhydrazine. Thus, the assay measures hydrogen atom donating ability and hence provides the measurement of antioxidant activity of a compound. All the experiments were performed in methanol. Five different concentrations 200, 160, 80, 40, and 20 μM of test compounds were used. In brief, 75 μL of different concentrations (200, 160, 80, 40, and 20 μM) of the test compounds were added to a 96-well plate. To this, 75 μL of DPPH (100 μM final concentration) solution was added. Finally, a 96-well microplate was incubated at 37 ºC for 25 min in a shaking water bath with moderate shaking. The absorbance was measured at 520 nm wavelength using a 96-well microplate reader (Synergy^{TM} HT, Bio-Tek Instruments, Inc. and Epoch 2 microplate reader, Bio-Tek Instruments, Inc.). A low absorbance indicates an effective free radical scavenging capacity. The reducing percentage (RP) of the DPPH was determined by the equation RP = [(absorbance of control − absorbance of the test)/ absorbance of control] X 100. All the experiments were performed in duplicate or triplicate.

***In-vitro* metal chelating assay with 7a and 4f**

The chelating studies were performed using a UV-vis spectrophotometer (Agilent Cary 60 UV-Vis spectrophotometer) wavelength ranging from 200 to 700 nm. Desired concentrations of the compounds (**7a** or **4f**) were dissolved in deionized water to make a 600 μM solution, and pH was monitored using a pH meter. Further, the compounds (**7a,** or **4f**) were diluted to make a 300 µM solution. The solution obtained was scanned under a UV-vis spectrophotometer in the range of 200-700 nm. Finally, $FeCl_3$ (1.2 mM) was dissolved in deionized water to prepare a colorless stock solution. The two solutions, $FeCl_3$ (600 µM), and compound (600 µM), were mixed in equal

proportions, and the pH was monitored and scanned with a UV-vis spectrophotometer. The pH of the solution was raised to 7.4 by adding diisopropylethylamine (50 μL DIPEA+ 950 μL DI water) to the solution, followed by UV scanning. The absorption spectra of each compound (300 μM) alone or in the presence of $FeCl_3$ (300 μM, final concentration) were recorded in the range of 200-700 nm for 30 min at 25 °C. The graph was plotted to compare the UV shift before and after the complexation. The metal chelation assay was performed in triplicate. The above samples were analyzed by the MS-MS technique to further confirm the stoichiometry of the complex.

***In-vitro* metal chelating assay with 23b**

The metal chelating studies were performed as described above with modifications. Briefly, the metal chelation was monitored spectrophotometrically using Epoch 2 microplate reader and Bio-Tek Instruments, Inc. Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$, CAS No. 10025-77-1) was purchased from Sigma Aldrich (USA). The stock solution of compound **23b** (600 μM) was prepared in extra pure methanol. Compound **23b** was then diluted in methanol to make a 300 μM solution and the solution was kept aside for absorbance reading on the next day from 300 to 700 nm. $FeCl_3.6H_2O$ was next dissolved in methanol to make a 600 μM light orange solution. The two solutions [**23b** (600 μM) and $FeCl_3.6H_2O$ (600 μM)] were mixed together in equal volume, which gave a light-yellow solution. Finally, the sample solution was incubated at 25 ºC for 24 h on a thermomixer with vigorous shaking at 1000 rpm. After 24 h, the solution color changed from yellow to blue and was subjected to UV scan. Finally, the pH of the solution was maintained to 7.4 by adding base diisopropylethylamine (DIPEA) (diluted with water), which produced a deep-blue color which was followed by a UV spectra scan. Each concentration was assayed in duplicate.

The molar ratio method was performed in order to determine the stoichiometry of the **23b-**Fe (III) complex by titrating the solution of compound **23b** with $FeCl_3$ solution (at different molar ratios).

The UV spectra were recorded at room temperature. The absorbance spectrum represents the formation of a new absorption band, indicating that the compound can interact with Fe (III) ions. Thereafter, the mole fraction of Fe (III) and absorbance of **23b** was plotted to obtain stoichiometry of the complex.

Amyloid-β aggregation studies

Aβ aggregation study was performed using atomic force microscopy with a NT-MDT Ntegra Prima Atomic Force Microscope (AFM). Amyloid β Protein Fragment 1-42 (CAS Number 107761-42-2) was purchased from Sigma Aldrich and Genscript. For the $A\beta_{1-42}$ aggregation inhibition experiment, the Aβ stock solution was diluted with phosphate buffer saline (pH = 7.4) to 0.79 μM before use. The $A\beta_{1-42}$working concentration was estimated through the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific instrument). A mixture of the peptide (50 μL, 0.39 μM, final concentration) with or without the test compound (50 μL, 0.79 μL final concentration in ratio 1:2) was incubated on an Eppendorf thermomixture C with the condensation-prevention top (Eppendorf, catalog No. 5382000015) with agitation at 1400 rpm, at 37 ºC for 10 days for the aggregation experiment.

Substrate preparation and plate casting

For AFM film generation, three samples were prepared (1) Fresh Aβ Protein (0.39 μM), (2) Aβ Protein alone (0.39 μM), (3) Aβ Protein with **7a** (in a 1:2 ratio), (4) Aβ Protein with **FA** (in a 1:2 ratio), and (5) Aβ Protein with DPZ (in a 1:2 ratio). The samples for the AFM imaging were prepared by the drop-casting method using a silicon dioxide substrate [free gift from Dr. Bhola Nath Pal, SMST, IIT (BHU)]. The plates were washed through ultrasonic agitation on a GT sonic, ultrasonic cleaner (AnTech, Product Code: VGT-1990QTS) by placing silicon dioxide plates on the substrate holding tray (made up of Teflon) into the beaker, followed by acetone washing for 5

min. After acetone washing, the substrate was rinsed with isopropyl alcohol using the same procedure as mentioned above. Thereafter, plates were subjected to drying in desiccators for 1 h. In plate casting, fresh $A\beta_{1-42}$ Protein, $A\beta_{1-42}$ alone, and protein $A\beta_{1-42}$ with compound sample (1: 2) were subjected to the substrate by placing 10 μL of the protein sample on the freshly washed silicon dioxide plates, which were dried at room temperature or placed under vacuum for AFM imaging. The images were visualized using the Nova Px image analysis software (NT-MDT Ntegra Prima, Russia).

Molecular docking studies against AChE and BChE

Molecular docking studies can provide valuable information on the mode of interaction of the synthesized ligands at the active site of AChE and BChE enzymes. Molecular docking studies were performed using Schrödinger software. X-ray crystal structures of AChE (PDBID: 4EY7) and BChE (PDB ID: 4BDS) enzymes were obtained from the Protein Data Bank (www.rscb.org) and proteins were prepared using the "protein preparation wizard" module implemented in the Schrodinger software [165, 181]. The energy was minimized using the OPLS3 force field. Validation and optimization of the docking protocol were performed by redocking the co-crystallized ligands into the active site of the enzymes (AChE and BChE). The 2D structures of compounds **4c**, **4f**, **4j**, **4n**, **7a**, **7b**, **10b**, DPZ, **FA**, and tacrine were sketched in the 2D sketcher module of Maestro, and 3D minimized using the LigPrep module of the Schrodinger software [141]. The active site of the proteins was defined by the centroid of the co-crystalized ligands present in the X-ray crystal structures of 4EY7 and 4BDS. Standard precision with flexible ligand sampling was used in the Glide docking. The Prime-MMGBSA module of Schrödinger was used to calculate the binding free energies of the best-docked complex of the compounds with the AChE and BChE proteins.

Molecular dynamics simulations

The binding stability and interaction profile of the docked complex of potent inhibitor **7a** were analyzed on AChE and BChE. For this study, a 30 ns molecular dynamics simulation was performed using the Desmond module of Schrödinger [182]. Solvation of the protein-ligand complex was performed using the TIP4P water model and a buffer distance of 10 Å between the box edge and atoms of the complex. The system was neutralized by adding 0.15 M NaCl. The system was energy minimized with a maximum of 20,000 steps, and a modified relaxation protocol was applied for equilibration of the system as described in our previous publication [183]. The constant NPT production run of 30 ns using a time step of 2 fs was performed with no constraints.

Molecular docking of 7a with Aβ_{42} protofibrils

The NMR 3D structure of Alzheimer's Aβ_{42} protofibrils was retrieved from the Brookhaven protein data bank having PDB ID 2BEG [184, 185]. Ligand molecule **7a** (**F24**) was drawn using a 2D sketcher and subsequently converted to 3D structure and minimized in the Schrödinger software package (Schrödinger, L.L.C., and New York, NY). Molecular docking of small-molecule **7a** (**F24**) to Aβ_{42} protofibrils was performed using the Achilles blind docking server available at (https://bio-hpc.ucam.edu/achilles/) which is a customized version of Autodock Vina. This customized tool performs an exhaustive series of docking all over the protein surface and finds the site with high binding affinity. Generally, a single Vina run of blind docking performs poor sampling across the whole protein surface, and this limitation is overcome by using this customized version of Autodock Vina [186].

Molecular dynamics simulation study of 7a with Apo-protofibril and protofibril-7a complex

All-atom molecular dynamics simulations were performed using the GROMACS v2018.8 software package using the CHARMM36 force field [187, 188]. Two systems, i.e., Apo-protofibril

and protofibril-F24 complex, were prepared and subjected to MD simulations. The official CHARMM General Force Field server CGenFF was used to create the topology files for small molecule **7a** [187, 189]. Water molecules were represented by the TIP3P water models [190]. Apo-protofibril and protofibril-**7a** (**F24**) complex was placed in a cubic box with a minimum of 1.0 nm distance between solute and box edges. The box was solvated with TIP3P water models, and the system having negative charge was neutralized by using Na^+ ions. The neutralized system was energy minimized using the steepest descent minimization method with 50000 steps. This minimization step removes the collision and incorrect geometry of structure among any atoms in the simulation system. Both NVT followed by NPT equilibration was performed for 100 ps using position restrained on protein and protein-ligand complex. The cutoff ratios of 1.2 nm for Coulomb and van der Waals potentials were used for the calculation of short-range nonbonded interactions. During the simulation, the pressure (1 atm) and temperature (310K) of the system were maintained by Parrinello-Rahman barostat and V-rescale thermostats, respectively [191, 192]. Long-range electrostatic interactions were calculated using the particle mesh Ewald method with a grid spacing of 0.16 nm [193]. Finally, well minimized and equilibrated systems were subjected to MD run for 200 ns. The simulation trajectory was analyzed using the various auxiliary tools available in GROMACS. The binding energy between the protofibril-**7a** (**F24**) complex and interchains were calculated using g_mmpbsa software [194].

Principal component analysis

PC analysis was carried out using Bio3D in the R software package [195, 196]. Cα coordinates of protofibril and protofibril-**7a** (**F24**) complex from 200ns MD trajectory were used as the input for analysis. The first step includes trajectory frame superposition in which each frame was aligned with respect to the initial frame. Then a covariance matrix was constructed, which captured the

degree of co-linearity of motions for each pair of Cα atoms. Subsequently, this covariance matrix was diagonalized, which provided a set of eigenvectors and their respective eigenvalues.

Molecular docking studies with 13k and 23b against AChE and BChE

The 3D crystal structures of *h*AChE in complex with DPZ (PDB ID-4EY7) and *h*BChE in complex with tacrine (PDB ID-4BDS) were retrieved from the Brookhaven protein data bank [22, 197]. Protein Preparation Wizard of Schrödinger software package (Schrödinger, LLC, New York, NY) was used to prepare both proteins. This step includes removal of water beyond 5 Å from the HET group, the addition of missing hydrogen, optimization of orientations of hydroxyl and amino groups, assignment of right bond orders, and the determination of ionization of amino acids using ProtAssign utility. The resulting structures were further subjected to restrained minimization with cutoff root mean square deviation (RMSD) of 0.3 Å. Finally, the prepared complexes were additionally used for molecular docking and MD simulation study. All the small molecules were drawn using 2D sketcher and were subjected to ligand preparation using the LigPrep module of Schrödinger software package (Schrödinger, LLC, New York, NY). The different possible ionization states for ligands were generated at the physiological pH (7.0 ± 2), and the OPLS3 force field was used to minimize the ligands. Two different grids, one for the AChE (4EY7) and the other for the BChE (4BDS) were prepared using the centroid of bound co-crystallized ligands. Both grids were standardized by re-docking the co-crystallized ligands *i.e.* DPZ and tacrine to AChE and BChE, respectively. Finally, docking of all ligands was performed by the Glide module of the Schrödinger software package (Schrödinger, LLC, New York, NY) using standard operating procedures with the extra precision (XP) protocol [198].

Molecular dynamics study of 13k and 23b with AChE and BChE

All-atom MD simulations were performed using the Desmond-v6.1 module of Schrödinger Software Package (Schrödinger, LLC, New York, NY) [199].The system builder panel was used to prepare the initial systems for MD simulations. The apo-AChE, apo-BChE, and all docked complexes were placed in a cubic box of 1.0 nm size. The boxes were solvated with TIP3P water models and charged systems were neutralized using counter ions (Na^+ or Cl^- ions) [190]. Ionic strength of 0.15 M was maintained by adding Na^+ and Cl^- ions to all the systems. Further, the solvated systems were minimized and equilibrated under NPT ensemble using the default protocol of Desmond. It includes a total of nine stages, among which there are two minimization and four short simulations (equilibration phase) steps [200, 201]. All minimized and equilibrated systems were subjected to MD run with periodic boundary conditions in NPT ensemble using OPLS_2005 force field parameter for 100 ns [202]. During the simulation, the pressure (1 atm) and temperature (300 K) of the systems were maintained by Martyna–Tobias–Klein barostat and Nose–Hoover Chain thermostat, respectively [203, 204]. The binding energy between the AChE/BChE and ligands (3k & 13b) was calculated using the inbuilt script thermal_mmgbsa.py [205]. The binding energy was calculated from the last 25 ns of trajectory at an interval of 50 ps for all four (AChE-**13k**, AChE-**23b**, BChE-**13k**, and BChE-**23b**) systems. The solvent accessibility surface area (SASA) of AChE/BChE in the presence of different ligands was also calculated using the script binding_sasa.py.

***In-vitro* parallel artificial membrane permeation assay (PAMPA) assay**

The blood-brain barrier penetration assay of the developed molecule was performed using the parallel artificial membrane permeation assay (PAMPA) described by Di *et al.*[171]. The commercially available molecules caffeine (CAS No. 58-08-2), testosterone (CAS No. 58-22-0),

imipramine (CAS No. 113-52-0), hydrocortisone (CAS No. 50-23-7), and corticosterone (CAS No. 50-22-6) served as controls and were purchased from TCI chemicals. Porcine brain lipid (PBL, CAS No. 86088-88-2, Lot No. 141101P-100MG-A-079) and *n*-dodecane (PBL, CAS No. 112-40-3) were purchased from Sigma Aldrich and Avra synthesis, India, respectively. The PAMPA kit (Cat # PAMPA 096, Lot No. BJ07A12), including the donor microplate (PVDF membrane, pore size 0.45 mm) and acceptor microplates, were purchased from Bioassay systems Pvt. Ltd. All the experiments were carried out in phosphate buffer saline (PBS, pH 7.4). The assay was carried out following the manufacturer protocol. Briefly, the filter membrane of the donor (top) plate was coated with BBB-specific lipid solution prepared by dissolving 5 mg of PBL (porcine brain lipid) in 250 μL of n-dodecane. Thus coated donor plate was dried for 30 minutes in the fume hood. The stock solutions (10mM) of test or controls were prepared in molecular biology grade DMSO. Thereafter, 500 μL of 500 μM test or controls was prepared by dissolving 25 μL of stock solution of test or controls in 475 μL of PBS (pH 7.4). Thereafter, 200 μL of equilibrium standard of each of the test compound or controls were prepared by dissolving 80 μL of 500 μM of test or control with 120 μL of PBS (pH 7.4). Finally, 250 μL blank control was prepared by dissolving 5 μL of DMSO in 245 μL of PBS (pH 7.4).

The acceptor plate wells were filled with 300 μL of PBS buffer. Next, 200 μL of each test molecule (500 μM), and controls (500 μM) were added in duplicate to the well of coated donor plate. Next, the donor plate was carefully placed over the acceptor plate wells to make a sandwich and incubated for 18h at 37°C in a moistened sealed bag to prevent evaporation. The test compound concentrations, controls, blank control, and equilibrium standards were determined by UV spectroscopy (Epoch 2 microplate reader, BioTek, U.S.A.) by adding 100 μL of the respective solutions to a 96-well plate. The absorbance spectrum from 200 nm to 500 nm in 10 nm intervals

was determined to determine the absorbance of the tested compounds. Each experiment was performed in at least duplicate. P_e value was calculated using the following expression: Pe = Cx - In (1- OD_A/OD_E) cm/s, where OD_A = absorbance of acceptor solution, OD_E is the absorbance of equilibrium standard, and if the experiment is running for 18 hrs incubation, then C = 7.72 x 10^{-6}. The results are given as the mean ± SD.

Cytotoxicity studies

Human neuroblastoma, SH-SY5Y cells (National Centre for Cell Science, Pune) were used for cytotoxicity studies. SH-SY5Y cells were cultured in 25 cm^2 tissue culture flasks and incubated in DMEM (Cell clone) and Ham's F12 (Sigma) media, (1:1) supplemented with penicillin-streptomycin (1X), plasmocin prophylactic (1X) and heat-inactivated fetal bovine serum (10%) at 37 °C in a humidified incubator with 5% CO_2 humidified atmosphere. The growth medium was replaced every third day and cells were passaged at 75% confluence.

Assessment of cell viability by MTT assay

The cytotoxicity of **7a** (**F24**) on SH-SY5Y cells was determined by MTT assay. SH-SY5Y cells were seeded at a concentration of 1×10^4 cells/well in 96-well plates and were incubated overnight for adherence. The compound, **7a** (**F24**) was added in SH-SY5Y cells at a concentration of 20, 10, 5, 2.5, 1, 0.1, and 0.01 μMin triplicates and incubated for 24 h at 5% CO_2 at 37°C. Cells without **7a** (**F24**) were used as control. Post incubation, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltratrazolium bromide (MTT) (Himedia) (5 mg/mL) was added. After four hours, 0.04 M HCl isopropanol was added to the medium having an MTT solution and incubated at 37°C for 1h in the dark. Absorbance was measured at 570 nm using a 96-well microplate reader (Synergy^{TM} HT, Bio-Tek Instruments, Inc.). Mean absorbance (OD_{570}) values were plotted against

different concentrations of **7a** (**F24**) used. Percent cell viability was calculated by using the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD value of the experimental sample (treated)}}{\text{Mean OD value of experimental control (untreated)}} \times 100$$

Percentage cytotoxicity was calculated by the following formula:

% cytotoxicity = 100% – % cell viability.

Effect of compound 7a on H_2O_2 induced cell death

The H_2O_2-induced cellular damage model of neurodegenerative disorder in SH-SY5Y cell line was established to evaluate the protective effect of **7a** (**F24**). The cells were seeded at a density of 1×10^4 cells/well into 96-well plates and incubated overnight. Next, the cells were treated with 100, 200, 400, 600, and 800 μM of H_2O_2 for 24 h, and control wells were cultured without any treatment. Following the treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added and incubated at 37 °C for 4 h. Next, the solution in the wells was replaced by the extraction buffer (0.04 N HCl in isopropanol), followed by additional incubation at 37°C for 1 h in the dark and the optical densities were measured at 570 nm using a multimode microplate reader (Synergy HT, Bio-Tek Instruments, Inc.). Each experiment was repeated at least in triplicate.

The MTT assay was also used to determine the protective effect of **7a** (**F24**) in H_2O_2-induced cell damage based on the evaluation of cell viability. The results were also confirmed by microscopic observation. The SH-SY5Y cells were seeded at a density of 1×10^4 cells/well into 96-well plates and incubated overnight. Next, the cells were grown in the presence or absence of different concentrations (1, 2.5, 5, 10, or 20 μM) of **7a** (**F24**) or DPZ for 24 h, then further co-incubated with 600 μM of H_2O_2 for another 24 h. The cells in the control group were cultured without any

treatment. Each experiment was repeated at least in triplicate. Post-treatment, MTT was added to all of the cells seeded wells and incubated at 37 °C for 4 h followed by a general procedure described above. Percent cell viability was calculated by the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD value of experimental sample (treated)}}{\text{Mean OD value of experimental control (untreated)}} \times 100$$

Morphological observations

SH-SY5Ycells for live-cell microscopy were seeded at a density of 1×10^6 cells/well into a 6-well plate to adhere for one day and pretreated with **7a** (**F24**) for 24 h, then further co-incubated with 600 μM H_2O_2 for another 24 h. The cells in the control group were cultured without any treatment. Cell morphology was obtained using a live-cell imager fluorescence microscope (EVOS FL cell imaging system, Life Technologies, USA).

Measurement of intracellular ROS generation in SH-SY5Y cells treated with H_2O_2

SH-SY5Y cells were seeded at a concentration of 1×10^4 cells/well in 96 well plates and incubated overnight. Next, the cells were grown in the presence or absence of different concentrations (1, 2.5, 5, 10, or 20 µM) of **7a** (**F24**) or DPZ for 24 h, then further co-incubated with 600 μM H_2O_2 for another 24 h. The cells in the control group did not receive any treatment. After adding5 µM, MitoSOX red (Lot # 2015529, Invitrogen, USA) to each well followed by incubation for 15 min in the dark, the generation of ROS was measured through fluorescence intensity at excitation wavelength 510 nm and emission at 580 nm using a multimode microplate reader (Synergy TM HT, Bio-Tek Instruments, Inc.).

Apoptosis assay in SH-SY5Y cells treated with H_2O_2 by the TUNEL method

SH-SY5Y cells were seeded (1×10^5 cells) in the presence or absence of different concentrations (1, 2.5, 5, 10, or 20 μM) of **7a** (**F24**) for 24 h and subsequently co-incubated with 600 μM H_2O_2

for another 24 h. The control group cells were cultured without any treatment, and the positive control group was treated with DNase. The staining for the TUNEL assay was done following the manufacturer's protocol (K191–001, EZClick™ TUNEL-in situ DNA Fragmentation/Apoptosis Assay Kit, BioVision). Cells were observed at 40X magnification under a fluorescence microscope (EVOS FL cell imaging system, Life Technologies, USA)

Protection against A$\beta_{1\text{-}42}$ cytotoxicity by 7a (F24)

SH-SY5Y cells were seeded at a concentration of 1×10^4 cells/well in 96 well plates and incubated overnight. Next, the cells were grown in the presence or absence of 5 μM of Amyloid $\beta_{1\text{-}42}$ for 55h followed by MTT assay as described earlier. The cell growth response to A$\beta_{1\text{-}42}$ was detected by measuring the absorbance at 570 nm on the multimode plate reader. Three replicates were performed for each experiment. Later, the neuroprotective effect of **7a** (**F24**) on cellular damage caused by A$\beta_{1\text{-}42}$ to SH-SY5Y cells was assessed. The SH-SY5Y cells were seeded at a density of 1×10^4 cells/well into 96-well plates and incubated overnight. SH-SY5Y cells were grown in the presence or absence of different concentrations (5 and 10 μM) of **7a** (**F24**) or DPZ for 24 h, then further co-incubated with 5 μM A$\beta_{1\text{-}42}$ for another 55 h. The cells in the control group were cultured without any treatment. Each experiment was repeated at least in duplicate. After 55 h incubation, MTT was added following the general procedure.

Assessment of cell viability by MTT assay

The cytotoxicity of **23b** on N2a cells was determined by MTT assay following our earlier publications. Briefly, N2a cells were seeded at a concentration of 1×10^4 cells/well in 96 well plates and were incubated overnight for adherence. The compound, **23b** was added in N2a cells at a concentration of 20, 10, 5, 2.5, 1, 0.1 and 0.01 μM in triplicates and incubated for 24h at 5% CO_2 at 37°C followed by MTT assay.

Effect of compound 23b on H_2O_2 induced cell death

The H_2O_2-induced cellular damage model of neurodegenerative disorder in n2a cell line was established to evaluate the protective effect of **23b**. The cells were seeded at a density of 1×10^4 cells/well into 96-well plates and incubated overnight. Next, the cells were treated with 100, 200, 400, 600, and 800 μM of H_2O_2 for 24 h, and control wells were cultured without any treatment. Following the treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added and incubated at 37 °C for 4 h. Next, the solution in the wells was replaced by the extraction buffer (0.04 N HCl in isopropanol), followed by additional incubation at 37°C for 1 h in the dark and the optical densities were measured at 570 nm using multimode microplate reader (Infinite® 200 PRO, TECAN.). Each experiment was repeated at least in triplicate. The MTT assay was also used to determine the protective effect of **23b** in H_2O_2-induced cell damage based on the evaluation of cell viability. The results were also confirmed by microscopic observation. The n2a cells were seeded at a density of 1×10^4 cells/well into 96-well plates and incubated overnight. Next, the cells were grown in the presence or absence of different concentrations (1, 2.5, 5, 10, or 20 μM) of **23b** for 24 h, then further co-incubated with 600 μM H_2O_2 for another 24 h. The cells in the control group were cultured without any treatment. Each experiment was repeated at least in triplicate. Post-treatment, MTT was added to all of the cells seeded wells and incubated at 37 °C for 4 h followed by a general procedure described above. Percent cell viability was calculated by the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD value of experimental sample (treated)}}{\text{Mean OD value of experimental control (untreated)}} \times 100$$

The median lethal dose (LD_{50}) determination and therapeutic screening of 7a in Drosophila

Fruit fly strains OregonR$^+$ (wild type), ey-GAL4/CyO and ey-GAL4>UAS-Aβ_{42} were obtained from Bloomington *Drosophila* stock center, Indiana, U.S.A. The stocks were cultured in standard

corn food media at 24±1°C in BOD (Biological Oxygen Demand) incubator, while $A\beta_{42}$ expressing flies were cultured at 28±1°C in BOD incubator. To determine the median lethal dose (LD_{50}) of **7a** (**F24**), age-matched wild-type flies of ten virgin females and ten males were crossed in separate vials containing food treated with a range of **7a** (**F24**) concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 mg/ml) for 15 days. The same numbers of wild-type flies were set up in untreated normal food vials as a control. The F1 progeny was screened for lethality, and LD_{50} was determined accordingly.

Similarly, the effect of **7a** (**F24**) was also tested against AD flies by setting up genetic crosses between eye tissue-specific ey-GAL4/CyO and UAS-$A\beta_{42}$ flies. In the case of $A\beta_{42}$ expressing AD flies, the nontoxic doses ranging from 0.2 to 0.8 mg/ml were administered to test the neuroprotective efficacy of **7a** (**F24**). For control, the same genotype flies were cultured on normal food media (without **7a** (**F24**)). All experiments were performed in triplicates. The $A\beta_{42}$ induced eye phenotypes of F1 generation flies were scored using stereo zoom binocular microscope, and statistical analysis was done using a one-way ANOVA test to see the significance of rescue by **7a** (**F24**) (*$p < 0.0132$).

Animal studies

Adult male Swiss albino mice, 6 weeks old and weighing 30 ± 2 g were obtained from the Central Animal House of the University and acclimatized in an animal room for 7 days (12:12 h light/dark cycle, temperature 25 ± 2°C) in the Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (Banaras Hindu University). Mice were supplied with commercial food pellets and tap water unless otherwise stated. All the experimental methods and procedures conducted in this study were in accordance with the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests

and Climate Change, Government of India, and approved by the Central Animal Ethical Committee of the University (Banaras Hindu University, Varanasi, India) (**Dean/2018/ CAEC/1189, dated 20.04.2019**).

Acute toxicity test

Compound **7a** was tested for acute oral toxicity as per OECD guidelines. Compound **7a** (**F24**) was administered in graded doses up to 500 mg/kg. Mice were observed regularly for the first 24 h to notice behavioral changes, seizures, or diarrhea and then observed for mortality upto14days. All the mice were sacrificed on the 14th day after drug administration and were microscopically examined for possible damage to the liver.

Scopolamine-induced amnesia model and Y maze test

Drugs and chemicals

Scopolamine hydrochloride (CAS No. 55-16-3) and donepezil hydrochloride (CAS No. 120011-70-3) were purchased from Sigma–Aldrich. All other reagents used in this study were obtained from commercial suppliers and used without further purification.

Drug preparation and treatment protocol

Scopolamine hydrochloride (3 mg/kg), DPZ (5 mg/kg) and compound **7a** suspensions (6.25, 12.5 and 25 mg/kg) were freshly prepared in 0.5% v/v tween 80. The animals were randomly allocated into eight groups (n=5) namely, (i)vehicle control, (ii) scopolamine 3 mg/kg, i.p., (iii) DPZ 5 mg/kg, (iv) **7a** 6.25 mg/kg, p.o., (v) **7a** 12.5 mg/kg, p.o., (vi) **7a** 25 mg/kg, p.o., (vii) **FA** 12.5 mg/kg, p.o., (viii) **FA** 12.5 mg/kg, p.o. Test compounds or DPZ were administered daily upto 7 days. On the seventh day, scopolamine was administered to groups ii, iii, iv, v, vi, vii and viii after

30 min of drug administration. The vehicle control group received only the vehicle. Then, all the animals were subjected to a Y-maze test 15 min after vehicle or scopolamine administration.

Y-maze test

The Y-maze test is a simple, rapid, and sensitive test for the evaluation of exploratory behavior and spatial working memory in rodents. The Y-maze is a three-arm horizontal maze separated apart by 120°. The three arms of the maze were designated A, B, and C. During experimentation, each mouse was placed at the end of one arm and allowed to move freely through the maze for 8-min, during which the number of times it made a full entry (entry of all four limbs) into each arm was recorded using a video camera. Later, the data was analyzed, and the number of spontaneous alternations was calculated. Spontaneous alternations occur when a mouse enters into three different arms in three consecutive entries (ABC, ACB, BAC, BCA, CAB and CBA). The percentage of alternation was then calculated using the equation: % spontaneous alternation (SA) = [(number of alternations/total arm entries) – 2] X 100.This measure of %SA reflects the short-term memory in mice.

Neurochemicals estimation and antioxidant property evaluation

After the completion of the Y-maze experiment, all mice according to their respective groups were sacrificed immediately through cervical dislocation, and whole brains were isolated from the skull and homogenized with a glass homogenizer in 5 mL of 12.5 mM sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 7000 rpm for 30 min at 4 °C. The supernatants were collected and utilized for estimations of different biochemical parameters.

The cholinergic biomarker levels (AChE/BChE) were determined by Ellman's colorimetric method with slight modification. Briefly, 100 μL of the supernatant was incubated with ATCI or

BTCI (15 mM of 100 μL) for 5 min. After that, 100 μL of 1.5 mM DTNB was added, and the absorbance was recorded at 415 nm, immediately.

The estimation of antioxidant parameter superoxide dismutase (SOD) was performed as per the reported protocol. All of the reagent for this assay was prepared in phosphate buffer saline (PBS pH = 7.4). Briefly, the reagent was prepared by dissolving 50 mM of anhydrous sodium carbonate (Na_2CO_3), 0.1 mM of ethylenediaminetetraacetic acid (EDTA), and 25 μM of nitro blue tetrazolium (NBT) in PBS (0.1 M, pH 7.4). For the experiment, 100 μL of prepared reagent, 25 μL of hydroxylamine hydrochloride ($NH_2OH \cdot HCl$), and 50 μL of supernatant were mixed thoroughly, and the absorbance was recorded at 570 for 3 min at regular intervals.

Catalase (CAT) is an enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen. The CAT activity was evaluated as per the reported protocol described by Sinha. The assay mixture consisted of 50μM of PBS (0.1 M; pH = 7.4), 50 μL of 800 mM of hydrogen peroxide (H_2O_2), 50 μM of brain supernatant, and 100 μL of dichromate/acetic acid solution (5% $K_2Cr_2O_7$/glacial acetic acid; 1:3 v/v). Briefly, in a 96-well plate, 50 μM of brain supernatant, 50 μM of PBS (0.1 M, pH = 7.4) and 50 μL of 800 mM of hydrogen peroxide (H_2O_2) were incubated at 37 °C for 1 min. After incubation, 150 μL of dichromate/acetic acid solution (coloring reagent) was added followed by boiling at 100ºC for 10 min. Finally, the absorbance was recorded at 570 nm using a 96-well microplate reader.

Malondialdehyde (MDA) assay was used to access antioxidant properties on lipid peroxidation in the brain homogenate. Briefly, 0.2 mL of 8.1% sodium lauryl sulfate (SLS), 1.5 mL of 20% glacial acetic acid, and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid (TBA) was added to the 0.2 mL of processed brain homogenate. The mixture was made up to 4.0 mL with deionized water and heated at 95°C for 60 min. After cooling with tap water, 5 mL of n-butanol and pyridine

mixture (15:1 v/v) and 1 mL of distilled water were added and centrifuged. The organic layer (200 μL) was separated out in a 96-well plate, and absorbance was measured at 532 nm using a 96-well microplate reader.

Morris water maze

Adult male Swiss Albino mice (22−27 g) were divided into six different groups (7 animals each) according to their treatment (i) normal control, (ii) vehicle-control, (iii) scopolamine, (iv) DPZ 1 mg/kg with scopolamine and (v) **7a** (**F24**) at 1 mg/kg with scopolamine, and (vi) **7a** (**F24**) at 2.5 mg/kg with scopolamine. Scopolamine hydrochloride (1.4 mg/kg) was administered intraperitoneally (i.p.) to the animals of all groups except the control and vehicle-control group, which received an equal volume of saline or 0.5% v/v tween, respectively. DPZ (1 mg/kg) and **7a** (**F24**) (1 and 2.5 mg/kg) were administered intraperitoneally (i.p.) 30 min before administration of scopolamine to the respective group of animals for 22 days. From 18-22nd days of the treatment period, learning and memory test were performed using the Morris water maze (MWM) test.

The MWM consisted of a circular water tub (48 " diameter, 30 " height, 210 GAL, 49 LBS capacity, and the platform dimensions with rod height: 12” squared base (L X W): 12 "× 12 " top square plate (L X W): 4 "× 4 " top round plate: 4 " diameter) was filled with water (25 ± 2 °C), and titanium dioxide (TiO_2) was used to make the water opaque. The pool was divided into four different quadrants, and a hidden platform was submerged 1 cm below the water surface in the fourth quadrant. A camera was installed above the pool and connected to a computer to record the swimming pathway. The animal was placed in the first quadrant facing the wall and allowed to swim for 60 s to find the hidden platform, and the latency to attain the platform became measured from day 18 to 22nd.Throughout the experiment, the temperature of the pool (25 ± 2 °C) and the area of the platform were maintained constant.

In-Vivo experiments

Animals

3 months old male C57BL6 mice, 25−30 gm each, were obtained from the Breeding unit of small animal Facility and acclimatized in an experimental small animal facility for 5 days (12 h light/dark cycle, temperature 25 ± 2 °C) at the National Institute of Immunology, New Delhi). Mice were supplied with commercial food granules and sterilized water unless otherwise stated. All the methods and procedures performed in this study were following the guidelines approved (IAEC# 565/20) by the Institutional Animal ethical committee of the National Institute of Immunology, New Delhi.

Drugs and chemicals

The compound **23b** was suspended in 20 μl DMSO and volume makes up by the phosphate-buffered saline (pH 7.4), and DPZ.HCl and scopolamine hydrochloride was dissolved in water to carry out the *in-vivo* studies.

Morris water maze

Adult *C57*BL6 mice (25−30 g) were divided into five different groups (7 animals each) according to their treatment (i) normal control, (ii) scopolamine, (iii) DPZ 0.5 mg/kg with scopolamine, (iv) **23b** at 5 mg/kg with scopolamine, and (v) **23b** at 10 mg/kg with scopolamine. Scopolamine hydrochloride (1 mg/kg) was administered intraperitoneally (*i.p.*) to the animals of all groups except the control group, which received an equal volume of PBS, respectively. Donepezil hydrochloride (0.5 mg/kg i.p.) and **23b** (5 and 10 mg/kg) was administered orally (*p.o.*) 30 min before administration of scopolamine to the respective group of animals for 14 days. From 10-14th days of the treatment period, learning and memory test were performed using the Morris water maze (MWM) test. It consisted of a circular tank of 100 cm in diameter. The tank was divided

into four quadrants and labelled as A, B, C, and D. The quadrant in which the platform is placed is known as target quadrant/zone. Before the actual trials, mice were trained to find the hidden platform, three trials per day for 4 days. During the training period, the mice were allowed to swim freely for 60 s to find the hidden platform and to show them that there is a place in a pool where they can come and rescue themselves. Upon the actual start of the testing, the transparent platform was placed 1.5 cm below the water level. Animals were given three trials per day for 4 days consecutively. The escape latency was recorded, which is defined as the total time is taken (sec) to reach the platform in 4 consecutive days. On 5th day, a probe test was performed in which the platform was removed, and the animal was allowed to swim freely for 60 s to calculate the total time spent in the target quadrant. All the monitoring was done using a video tracking system attached to the computer and recorded with software. Data were analyzed using ANY maze software (Stoeling Co., USA). Throughout the experiment, the temperature of the pool (25 ± 2 °C) and the area of the platform were maintained constant.