# Chapter 4

## Development of Pyrazole and Spiropyrazoline Analogs

### 4.1 Experimental Work

#### 4.1.1 Rationale of drug design & *in-silico* optimization

Fragment-based drug discovery (FBDD) is a structure-based approach used to obtain lead compounds for the targets of biological interest. Screening of low-molecular-weight fragment libraries as a potential source of small, efficient lead identification has become a standard rationale for the drug discovery operations. Fragments, which are low molecular weight (MW< 250Da), small organic molecules, have low affinity (100 μM−10 mM) for binding to the target and are further embellished, grown and linked to create high affinity lead compounds [Shang et al. 2014, Wasko et al. 2015].

In-house available small molecular fragments were screened based on the cholinesterase interactions and further optimized through *in-vitro* experiments. AChE plays a crucial role in rapid hydrolysis of acetylcholine, in the central and peripheral nervous system. It is also involved in non-cholinergic mechanism related to neurodegeneration as is able to accelerate the amyloid β peptide assembly in to aggregates.

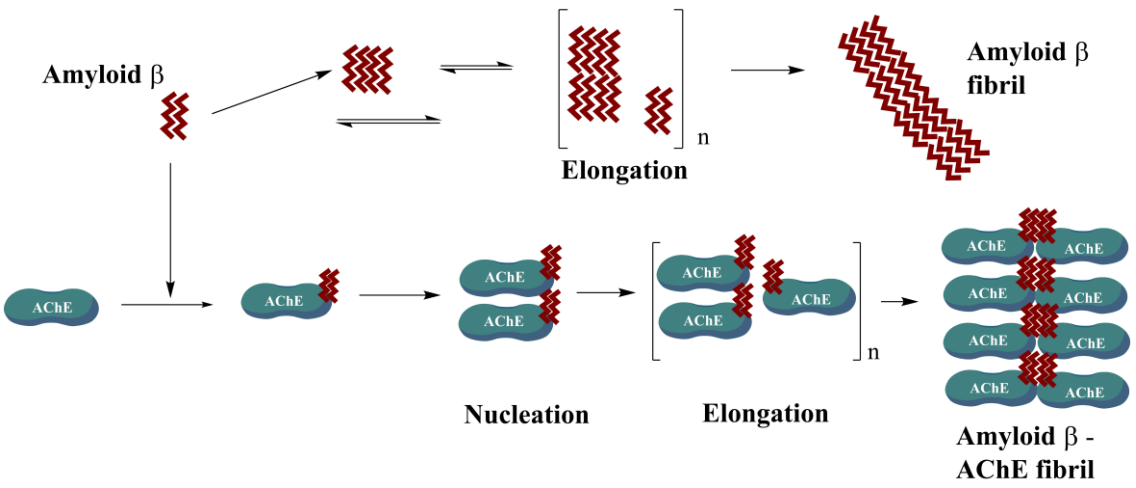



**Figure 4.1.** Role of AChE in the formation of amyloid fibrils.

The incorporation of AChE occurs at early steps of amyloid β aggregation, acting as a nucleation factor or seed, then it acts in the elongation of the amyloid fibrils. The design

of compounds was made to identify an ideal drug candidate, should inhibit the enzymes; produce potent activity against amyloid β aggregation and cross BBB.

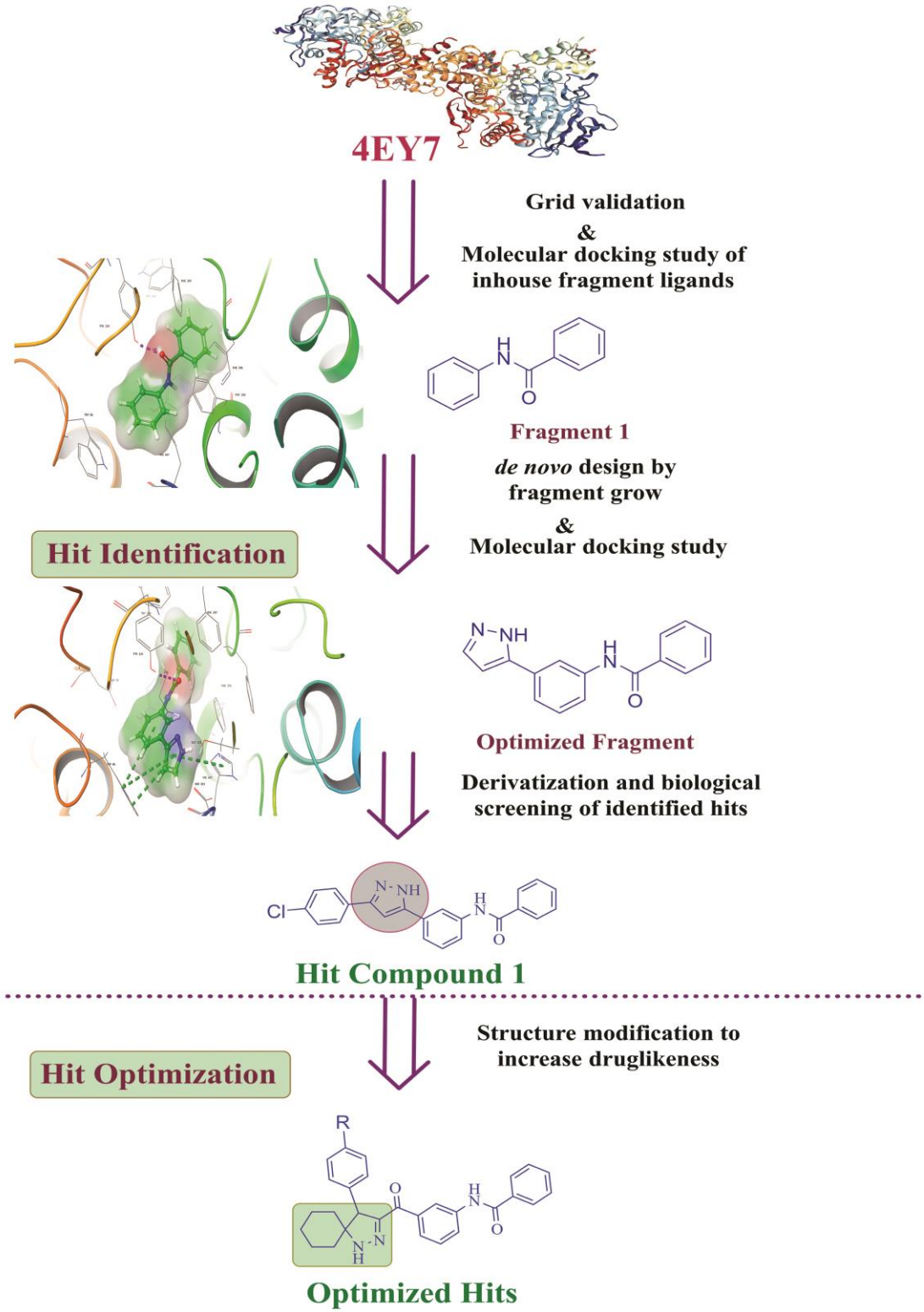



**Figure 4.2.** Overview of drug design process for pyrazole and spiropyrazoline analogs.

### 4.1.2 *In-silico* studies

#### 4.1.2.1 Seed growing

After selecting the fragments as seed structures, LigBuilder 2 was used to ameliorate these seeds in a stepwise manner. The PDB 4EY7 active site was identified using Cavity module. This module extracts interaction sites in binding site, which was used by Build module to produce various molecules using the seed fragments. The build module uses genetic algorithm to evolve various molecules, which were then scored and the better molecules were subsequently taken as seeds for generation of next level molecules. To make the optimization direction was reasonable and the molecular weight increase was restricted within 150 Da in the fragment growing process. The obtained ligands were subjected to the next round of optimization. The potent ligands were expected to be obtain through multiple optimization processes.

#### 4.1.2.2 Molecular docking

*In-silico* docking simulation protocols were performed using Schrödinger Glide module in Schrödinger Suite 10.5.014 MM Share Version 3.3.014 Release 2018-1. The ligands were prepared using LigPrep module. The minimum energy conformers of ligands were generated using OPLS2005 force field. Human acetylcholinesterase (PDB Code: 4EY7) was refined and processed using Protein Preparation Wizard module. The structure of the protein was further optimized using PropKa method at default pH value 7.0, and restrained minimization was performed for heavy atoms to RMSD 0.30 Å. Receptor Grid was generated surrounding the active binding pocket of the co-crystallized ligand DNZ. The prepared grid and docking simulation protocols of Glide (Grid-Based Ligand Docking with Energetics) extra precision (XP) mode were validated by re-docking DNZ. All other parameters of Glide module were maintained at their default values. The

docking results were studied using the Glide XP visualizer module to gain insights of the interactions of ligands with the amino acid residues.

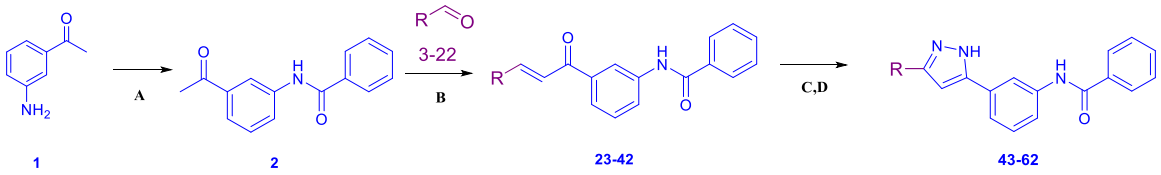
### 4.1.3 Synthesis and characterization

All commercially available chemicals were purchased from sigma Aldrich, TCI Co., Ltd, and Avra synthesis Pvt.Ltd. The solvents used for the study were dried and were used anhydrous unless otherwise stated. Experiments were carried out in oven-dried glassware's under dry $N_2$ atmosphere and standard vacuum techniques were used. Purifications were carried out by using column chromatography on silica gel 60 (Avra), particle size = 0.140−0.25 mm (60−120 mesh), as the stationary phase. All reactions were monitored on silica gel $F_{254}$ TLC aluminium sheets (Merck) and ultraviolet light (254 nm) or iodine vapors were used for visualization of spots. Melting points were determined on automated melting point apparatus (Bamstead Electrothermal, UK). All the intermediates and target compounds were characterized by $^{1}$H NMR, $^{13}$C NMR and Mass Spectrometry. NMR spectra were recorded in Bruker-500 ($^{1}$H 500 MHz, $^{13}$C 125.8 MHz) instrument using $CDCl_3$ and DMSO-$d_6$ as solvents. Chemical shift was measured in the ppm (δ) and coupling constant (*J*) was measured in Hz. $^{1}$ H NMR spectra are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublet, td = triplet of doublet, bs = broad singlet), coupling constant (*J*) in Hertz (Hz), integration, and proton assignment. Mass spectrometric analysis was performed using Waters Q-TOF premier-HAB213 instrument equipped with APCI and ESI multimode ionization source. Optical rotation was measured on a Fisher scientific, model ADP-45 automatic Polarimeter using a light-emitting diode (LED) lamp, emitting light at a wavelength of 589 nm and a 1 dm polarimetric tube. Specific rotation $[\propto]_{32.0}^{589}$, was calculated using the equation: $\alpha/lc$, where $\alpha$ is the observed optical rotation $\propto_{32.0}^{589}$, $l$ is the path length of the cell in dm, and

*c* is the concentration of test compound in g/ml. Differential scanning calorimetric analysis was performed by using heat flux type Shimadzu DSC-60 plus instrument with Chromel-Alumel thermocouple detector. X-ray data collection was performed with Bruker D8 VENTURE Kappa Apex III CMOS PHOTON 100 diffractometer equipped with graphite monochromated Mo (Kα) (λ = 0.71073 Å) radiation. The structures were solved by SHELXT-2014/5 (Sheldrick, 2014) and refined by full-matrix least squares techniques using SHELXL-2014, (Sheldrick, 2014) computer program. Molecular graphics were drawn by using ORTEP3 (Farrugia, 1997).

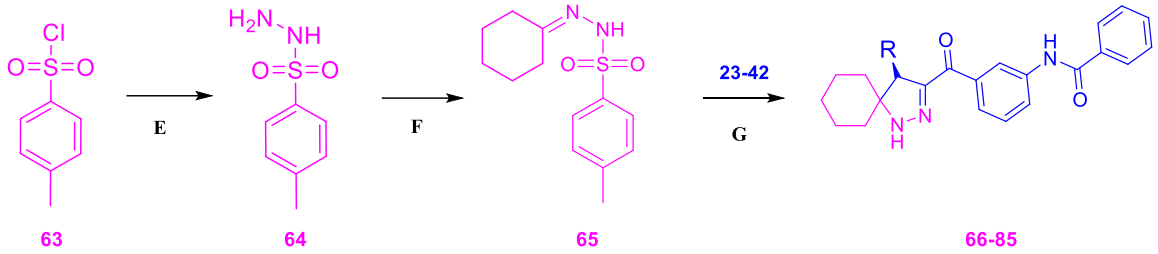
Purity of the compounds was determined by high-performance liquid chromatography (HPLC-Agilent 1260 infinity II Quaternary LC). Isocratic mobile phase was delivered by quaternary pump with flow rate of 1 ml/min. The mobile phase composition was phase A (Water) and phase B (methanol) in ratio of 1:9. 5 µL samples were injected into the HPLC column through auto sampler. Diode-array detectors (DAD, HS G7115A) detector was used at 310 nm for the detection of the compounds. Agilent ZORBAX Eclipse plus C8 column (5µm, 4.6 × 250 mm) was used. The purity of the compounds was found to be above 99%.

**4.1.3.1 Scheme 1. Synthesis of 3,5-diaryl-1H-pyrazole derivatives**




Reagents and conditions: (A) Benzoyl chloride 1.05 eq, triethylamine (1.05 eq), EtOAc, Rt, 6 h; (B) Aromatic aldehyde (**3-22),** (1.0 eq), 1M NaOH 1ml, EtOH, 25°C, 6 h; (C) Hydrazine hydrate (5.0 eq), EtOH, reflux, 2 h, then catalytic $I_2$ , DMSO, 110°C, 1.5 h.

### 4.1.3.2 Scheme 2. Synthesis of spiropyrazolines derivatives




| Comp. no | 03,23 43,66 | 04,24 44,67 | 05,25 45,68 | 06,26 46,69 | 07,27 47,70 | 08,28 48,71 | 09,29 49,72 | 10,30 50,73 | 11,31 51,74 | 12,32 52,75 |
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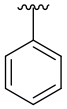
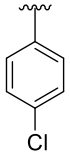
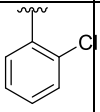
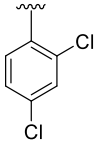
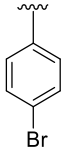
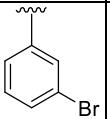
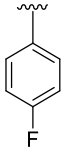
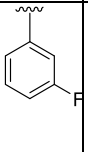
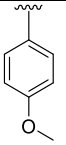
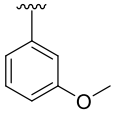
| R |  |  |  |  |  |  |  |  |  |  |

| Comp. no | 13,33 53,76 | 14, 34 54,77 | 15,35 55,78 | 16,36 56,79 | 17,37 57,80 | 18,38 58,81 | 19,39 59,82 | 20,40 60,83 | 21,41 61,84 | 22,42 62,85 |
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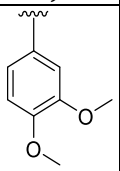
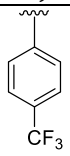
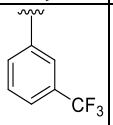
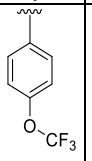
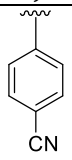
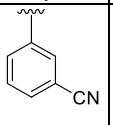
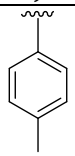
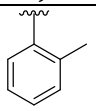
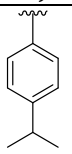
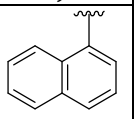
| R |  |  |  |  |  |  |  |  |  |  |

Reagents and conditions: (D) Hydrazine hydrate (1.5 eq), THF, 0°C, 30min; (E) Cyclohexanone (1.0 eq), MeOH, 60°C, 1 h; (F) **23-42** (0.5 eq), $CS_2CO_3$ (1.0 eq), 1,4-dioxane, reflux, 2 h.

### 4.1.3.3 Synthesis of N-(3-acetylphenyl)benzamide (2)

Compound **2** was synthesized according to a previously reported method [Ueda and Nagasawa 2009] and was obtained as off white solid. yield - 94.6%, M.P.- 108-109 °C,$^{1}$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 10.46 (s, 1H. amide), 8.38 (s, 1H, acetylphenyl $C_2$), 8.09 (d, $J$ = 8 Hz ,1H, acetylphenyl $C_6$), 8.00 (d, $J$ = 7.5 Hz, 2H, benzamide $C_2$, $C_6$), 7.73 (d, $J$ = 8Hz,1H, acetylphenyl $C_4$), 7.63-7.60 (m, 1H, benzamide $C_4$), 7.57-7.50 (m, 3H, benzamide $C_3$, $C_5$ & acetylphenyl $C_5$), 2.59 (s, 3H, methyl). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 198.2 (-C=O, acetyl), 166.52 (-C=O, amide), 139.64 (acetylphenyl $C_3$),

138.26 (acetylphenyl$C_1$), 135.42 (benzamide $C_1$), 131.57 (acetylphenyl $C_5$), 129.62 (benzamide $C_4$), 128.76 (benzamide $C_3$, $C_5$), 127.23 (benzamide $C_2$, $C_6$), 125.53 (acetylphenyl $C_6$), 123.27 (acetylphenyl $C_4$), 119.53 (acetylphenyl $C_2$), 27.22 (-$CH_3$, methyl).

**4.1.3.4 General procedure for the synthesis of chalcone derivatives (23-42)**

To a solution of compound **2** (1.0 eq) in ethanol (10ml) aromatic aldehyde (**3-22**, 1.0 eq) and 1M NaOH solution (1ml) were added at below 25°C. The reaction mixture was stirred at room temperature for 6 h. After completion of the reaction (monitored by TLC), reaction mixture was kept under refrigeration. Then the precipitated compound was filtered off and washed with ice cold ethanol (8 to 10ml) to afford titled compound.

*N-(3-cinnamoylphenyl)benzamide* **(23)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **3** and was obtained as a white solid, yield - 89%, M.P.- 115-116 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.51 (s, 1H, amide NH), 8.48 (s, 1H, phenyl $C_2$), 8.16 (d, $J$ = 7.5 Hz, 1H, β =CH ), 8.03 (d, $J$ = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.96 (d, $J$ = 7.5 Hz, 1H, phenyl $C_6$ ), 7.89 - 7.87 (m, 3H, phenyl $C_4$, $C_5$, benzamide $C_4$), 7.80 (d, $J$ = 16 Hz, 1H, α =CH), 7.63 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, cinnamoyl $C_2$, $C_6$), 7.47 (s, 3H, cinnamoyl $C_3$, $C_4$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 189.64 (-C=O, cinnamoyl), 166.24 (-C=O, amide), 144.64 (β =CH), 140.17 (phenyl $C_3$), 138.51 (phenyl $C_1$), 135.09 (cinnamoyl $C_1$), 132.25 (benzamide $C_1$), 131.18 (benzamide $C_4$), 129.64 (benzamide $C_3$, $C_5$), 129.45 (cinnamoyl $C_3$, $C_5$), 129.30 (Cinnamoyl $C_2$, $C_6$), 128.92 (benzamide $C_2$, $C_6$), 128.19 (phenyl $C_6$, cinnamoyl $C_4$), 125.36 (phenyl $C_5$), 124.47 (phenyl $C_4$), 122.63 (α =CH), 120.58 (Phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{17}NO_2$: 327.38, found – 328.4 (M+1).

*N-(3-(3-(4-chlorophenyl)acryloyl)phenyl)benzamide* **(24)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **4** and was obtained as a white solid, yield - 93%, M.P.- 105-106 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.47 (t, *J* = 2 Hz, 1H, phenyl $C_2$), 8.15 (d, *J* = 7 Hz, 1H, β =CH ), 8.02 - 8.00 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.97 - 7.90 (m, 4H, benzamide $C_2$, $C_6$, phenyl $C_4$, $C_6$), 7.78 (d, *J* = 15.5Hz, 1H,α =CH), 7.64 - 7.53 (m, 6H, chlorophenyl $C_2$, $C_6$, $C_3$, $C_5$, benzamide $C_4$, phenyl $C_5$ ). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.50 (-C=O, acryloyl), 166.21 (-C=O, amide), 143.14 (β =CH), 140.18 (phenyl $C_3$), 138.40 (phenyl $C_1$), 135.64 (phenyl $C_6$), 135.07 (chlorophenyl $C_4$), 134.09 (chlorophenyl $C_1$), 132.25 (benzamide $C_1$), 131.01 (benzamide $C_4$), 129.63 (benzamide $C_3$, $C_5$), 129.47 (chlorophenyl $C_2$, $C_6$), 128.91 (chlorophenyl $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.46 (phenyl $C_5$), 124.56 (phenyl $C_4$), 123.34 (α =CH), 120.56 (Phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}ClNO_2$: 361.83, found – 362.15 (M+), 364.12 (M+2).

*N-(3-(3-(2-chlorophenyl)acryloyl)phenyl)benzamide* **(25)**: This compound was also synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **5,** obtained as an off white solid, yield - 92%, M.P.- 119-120 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.50 (s, 1H, amide NH), 8.48 (s, 1H, phenyl $C_2$), 8.20 (d, *J* = 7.5 Hz, 1H, chlorophenyl $C_3$), 8.16 (d, *J* = 8 Hz, 1H, phenyl $C_6$), 8.08 (d, *J* = 15.5 Hz, 1H, β =CH), 8.02 (d, *J* = 7.5 Hz, 2H, benzamide $C_2$, $C_6$), 7.98 (d, *J* = 7.5 Hz, 1H, benzamide $C_4$ ), 7.95 (d, *J* = 15.5 Hz, 1H, α =CH), 7.63 - 7.54 (m, 5H, benzamide $C_3$, $C_5$, phenyl $C_4$, $C_5$, chlorophenyl $C_6$), 7.51 - 7.45 (m, 2H, chlorophenyl $C_4$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.41 (-C=O, acryloyl), 166.24 (-C=O, amide), 140.23 (β =CH), 139.15 (phenyl $C_3$), 138.20 (phenyl $C_1$), 135.06 (chlorophenyl $C_2$), 134.83 (benzamide $C_1$), 132.75 (chlorophenyl $C_1$), 132.53 (phenyl $C_6$), 132.25 (benzamide $C_4$) ,

130.55 (chlorophenyl $C_3$), 129.68 (chlorophenyl $C_4$), 129.01 (chlorophenyl $C_6$), 128.91 (benzamide $C_3$, $C_5$), 128.22 (chlorophenyl $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.63 (phenyl $C_5$), 125.38 (phenyl $C_4$), 124.63 (α =CH), 120.60 (Phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}ClNO_2$: 361.83, found – 362.05 (M+) 363.7 (M+2).

*N-(3-(3-(2,4-dichlorophenyl)acryloyl)phenyl)benzamide* **(26)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **6** and was obtained as an off white solid, yield - 94%, M.P.- 126-127 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.47 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.22 (d, *J* = 8.5Hz, 1H, β =CH), 8.15 - 8.13 (dd, *J* = 8Hz, 1.5Hz, 1H, phenyl $C_6$), 8.01 - 7.99 (m, 2H, benzamide $C_2$, $C_6$), 7.97 - 7.96 (m, 3H, phenyl $C_4$, benzamide $C_4$, α =CH), 7.74 (d, *J* = 2Hz, 1H, dichlorophenyl $C_5$), 7.62 - 7.53 (m, 5H, benzamide $C_3$, $C_5$, phenyl $C_5$, dichlorophenyl $C_3$, $C_6$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 188.77 (-C=O, acryloyl), 165.75 (-C=O, amide), 139.77 (β =CH), 137.62 (phenyl $C_3$), 137.40 (phenyl $C_1$), 135.67 (dichlorophenyl $C_2$), 135.15 (benzamide $C_1$), 134.57 (phenyl $C_5$), 131.77 (benzamide $C_4$), 131.35 (dichlorophenyl $C_1$), 129.78 (dichlorophenyl $C_6$), 129.53 (dichlorophenyl $C_3$), 129.18 (dichlorophenyl $C_5$), 128.43 (benzamide $C_3$, $C_5$), 127.97 (phenyl $C_6$), 127.70 (benzamide $C_2$, $C_6$), 125.41 (dichlorophenyl $C_4$), 125.24 (phenyl C4), 124.21(α =CH), 120.12 (phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{15}Cl_2N_2O_2$: 396.27, found – 396.5 (M+), 398.6 (M+2).

*N-(3-(3-(4-bromophenyl)acryloyl)phenyl)benzamide* **(27)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **7,** obtained as a whitish brown solid, yield - 92%, M.P.- 105-106 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.47 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.15 (dd, *J* = 8 Hz, 1.5 Hz, 1H, phenyl $C_6$), 8.02 (s, 1H, benzamide $C_2$), 8.01 (s, 1H, benzamide $C_6$), 7.96 (d, *J* = 8Hz, 1H, benzamide $C_4$), 7.93 (d, *J* = 15.5Hz, 1H, β

=CH), 7.86 (d, *J* = 8.5Hz, 2H,bromophenyl $C_3$, $C_5$), 7.76 (d, *J* = 15.5Hz, 1H, α =CH), 7.68 (d, *J* = 8.5Hz, 2H, bromophenyl $C_2$, $C_6$ ), 7.64 - 7.54 (m, 4H, phenyl $C_4$, $C_5$, benzamide $C_3$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.52 (-C=O, acryloyl), 166.21 (-C=O, amide), 143.22 (β =CH), 140.18 (phenyl $C_3$), 138.40 (phenyl $C_1$), 135.07 (benzamide $C_1$), 134.42 (beomophenyl $C_1$), 132.40 (bromophenyl $C_3$, $C_5$), 132.24 (phenyl $C_5$), 131.21 (benzamide $C_3$, $C_6$), 129.63 (benzamide $C_4$), 128.91 (bromophenyl $C_2$, $C_6$), 128.18 (benzamide $C_2$, $C_6$), 125.47 (phenyl $C_6$), 124.55 (phenyl $C_4$), 124.51 (bromophenyl $C_4$), 123.41 (α =CH), 120.58 (Phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}NO_2$: 406.28, found – 405.9 (M+) 407.9 (M+2).

*N-(3-(3-(3-bromophenyl)acryloyl)phenyl)benzamide* **(28)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **8,** obtained as whitish brown solid, yield - 94%, M.P.- 109-110 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.47 (s, 1H, amide NH), 8.46 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.19 (s, 1H, bromophenyl $C_2$), 8.15 - 8.13 (dd, *J* = 8Hz, 1.5Hz, 1H, phenyl $C_6$), 8.01 - 7.94 (m, 4H, benzamide $C_2$, $C_6$, β =CH, benzamide $C_4$), 7.87 (d, *J* = 8Hz, 1H, phenyl $C_4$ ), 7.75 (d, *J* = 15.5Hz, 1H, α =CH), 7.65 - 7.54 (m, 5H, benzamide $C_3$, $C_5$, bromophenyl $C_4$, $C_5$, $C_6$), 7.44 (t, *J* = 8Hz, 1H, bromophenyl $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.00 (-C=O, acryloyl), 165.72 (-C=O, amide), 142.31 (β =CH), 139.68 (phenyl $C_3$), 137.83 (bromophenyl $C_1$), 137.16 (phenyl $C_1$), 134.58 (benzamide $C_1$), 133.07(phenyl $C_5$), 131.75 (bromophenyl $C_2$), 130.96 (benzamide $C_4$), 130.82 (bromophenyl $C_4$), 129.12 (bromophenyl $C_5$), 128.42 (benzamide $C_3$, $C_5$), 128.12 (bromophenyl $C_6$), 127.69 (benzamide $C_2$, $C_6$), 125.08 (phenyl $C_6$), 124.20 (phenyl $C_4$), 123.66 (bromophenyl $C_3$) ,122.39 (α =CH), 120.13 (phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}NO_2$: 406.28, found – 405.9 (M+) 407.9 (M+2).

*N-(3-(3-(4-fluorophenyl)acryloyl)phenyl)benzamide* **(29)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **9,** obtained as a white solid, yield- 96%, M.P.- 126-127 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.50 (s, 1H, amide NH), 8.47 (s, 1H, phenyl $C_2$), 8.15 (d, *J* = 8Hz, 1H, phenyl $C_6$), 8.02 - 7.95 (m, 5H, benzamide $C_2$, $C_6$, β =CH, fluorophenyl $C_2$, $C_6$), 7.87 - 7.76 (m, 2H, α =CH, benzamide $C_4$), 7.63 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, phenyl $C_4$, $C_5$), 7.33 (t, *J* = 9Hz, fluorophenyl $C_3$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.52 (-C=O, acryloyl), 166.21 (-C=O, amide), 164.92 (fluorophenyl $C_4$), 143.39 (β =CH), 140.17 (phenyl $C_3$), 138.49 (phenyl $C_1$), 135.07 (benzamide $C_1$), 132.24 (phenyl $C_5$), 131.72 (benzamide $C_4$), 131.65 (fluorophenyl $C_1$), 129.61 (fluorophenyl $C_2$, $C_6$), 128.91 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.38 (phenyl $C_6$), 124.51 (phenyl $C_4$), 122.51(α =CH), 120.56 (phenyl $C_2$), 116.54 (fluorophenyl $C_3$), 116.37 (fluorophenyl $C_5$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}FNO_2$: 345.37, found – 346.52 (M+ 1).

*N-(3-(3-(3-fluorophenyl)acryloyl)phenyl)benzamide* **(30)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **10,** obtained as a white Solid, yield - 91%, M.P.- 128-129 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.47 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.16 - 8.14 (m, *J* = 8, 2, 1Hz, phenyl $C_6$), 8.02 - 7.95 (m, 4H, benzamide $C_2$, $C_6$, benzamide $C_4$,β =CH), 7.86 - 7.83 (dt, *J* = 10, 2Hz, 1H, phenyl $C_5$), 7.79 (d, *J* = 15.5Hz, 1H, α =CH), 7.72 (d, *J* = 7.5Hz, phenyl $C_4$), 7.64 - 7.49 (m, 5H, benzamide $C_3$, $C_5$, fluorophenyl $C_2$, $C_4$, $C_5$), 7.32 - 7.29 (td, *J* = 8.5, 2Hz, 1H, fluorophenyl $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.54 (-C=O, acryloyl), 166.21 (-C=O, amide), 163.95 (fluorophenyl $C_3$), 143.13 (β =CH), 140.19 (phenyl $C_3$), 138.32 (fluorophenyl $C_1$), 137.73 (phenyl $C_1$), 135.06 (benzamide $C_1$), 132.25 (phenyl $C_5$), 131.41 (benzamide

$C_4$), 131.34 (fluorophenyl $C_5$), 129.64 (phenyl $C_6$), 128.92 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.96 (fluorophenyl $C_5$), 125.55 (fluorophenyl $C_6$), 124.66 (phenyl $C_4$), 124.08 (α =CH), 120.60 (phenyl $C_2$), 117.88 (fluorophenyl $C_4$), 115.27 (fluorophenyl $C_2$); MS (ESI-): *m/z* calculated for $C_{22}H_{16}FNO_2$: 345.37, found – 344.3 (M-1).

*N-(3-(3-(4-methoxyphenyl)acryloyl)phenyl)benzamide* **(31)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **11,** obtained as an off white solid, yield- 91%, M.P.- 125-126 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.48 (s, 1H, amide NH), 8.45 (s, 1H, phenyl $C_2$), 8.14 (dd, *J* = 8 Hz,1.5 Hz, 1H, β =CH ), 8.02 (s,1H, benzamide $C_2$), 8.01 (s, 1H, benzamide $C_6$), 7.93 (d, *J* = 8Hz, 1H, α =CH), 7.86 (d, *J* = 8.5Hz, 2H, methoxyphenyl $C_2$, $C_6$), 7.75 (d, *J* = 2Hz, 2H, phenyl $C_4$, $C_5$), 7.63 - 7.54 (m, 4H, benzamide $C_3$,$C_6$,$C_4$, Phenyl $C_6$), 7.04 (d, *J* = 9Hz, 2H, methoxyphenyl $C_3$, $C_5$ ), 3.83 (s, 3H, -OMe). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.44 (-C=O, acryloyl), 166.19 (-C=O, amide), 161.91 (methoxyphenyl $C_4$), 144.65 (β =CH), 140.11 (phenyl $C_3$), 138.81 (phenyl $C_1$), 135.10 (benzamide $C_1$), 132.23 (phenyl $C_5$), 131.23 (benzamide $C_4$), 129.56 (benzamide $C_3$, $C_5$), 128.91 (methoxyphenyl $C_2$, $C_6$), 128.18 (benzamide $C_2$, $C_6$), 127.73(methoxyphenyl $C_1$), 125.12 (phenyl $C_6$), 124.34 (phenyl $C_4$), 120.49 (α =CH), 120.07 (phenyl $C_2$), 114.94 (methoxyphenyl $C_3$, $C_5$), 55.87 (-OMe); MS (ESI+): *m/z* calculated for $C_{23}H_{19}NO_3$: 357.41, found – 358.1 (M+1), 359.2 (M+2).

*N-(3-(3-(3-methoxyphenyl)acryloyl)phenyl)benzamide* **(32)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **12,** obtained as a white solid, yield - 93%, M.P.- 127-128 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.48 (s, 1H, amide NH), 8.47 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.16 - 8.14 (m, *J* = 8 Hz, 1.5 Hz, 1H, phenyl $C_6$), 8.02 (m, 2H, benzamide $C_2$, $C_6$ ),

7.97 - 7.96 (dt, 1H, benzamide $C_4$), 7.90 - 7.87 (d, *J* = 15.5 Hz, 1H, β =CH), 7.77 - 7.74 (d, *J* = 16 Hz, α =CH), 7.64 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, phenyl $C_4$, $C_5$) 7.48 - 7.37 (m, 2H, methoxyphenyl $C_2$,$C_6$), 7.40 (t, *J* = 8Hz, methoxyphenyl $C_5$), 7.06 - 7.04 (m, *J* = 1H, methoxyphenyl $C_4$), 3.84 (s, 3H, -OMe). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.68 (-C=O, acryloyl), 166.21 (-C=O, amide), 160.16 (methoxyphenyl $C_3$), 144.62 β =CH), 140.17 (phenyl $C_3$), 138.51 (phenyl $C_1$), 136.53 (methoxyphenyl $C_1$), 135.09 (benzamide $C_1$), 132.23 (phenyl $C_5$), 130.46 (benzamide $C_4$), 129.61 (methoxyphenyl $C_5$), 128.91 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.38 (phenyl $C_6$), 124.54 (phenyl $C_4$), 122.95 (α =CH), 121.97 (methoxyphenyl $C_6$), 120.57 (phenyl $C_2$), 117.16 (methoxyphenyl $C_4$), 114.02 (methoxyphenyl $C_2$), 55.79 (-OMe); MS (ESI+): *m/z* calculated for $C_{23}H_{19}NO_3$: 357.41, found – 358.4 (M+1) 359.2 (M+2).

*N-(3-(3-(3,4-dimethoxyphenyl)acryloyl)phenyl)benzamide* **(33)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **13,** obtained as an off white solid, yield - 95%, M.P.- 122-123 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.48 (s, 1H, amide NH), 8.43 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.14 - 8.12 (dd, *J* = 8Hz, 1.5Hz, 1H, phenyl $C_6$), 8.02 (d, *J* = 7Hz, 2H, benzamide $C_2$, $C_6$), 7.96 (d, *J* = 8Hz, 1H, β =CH), 7.80 – 7.72 (m, 2H,α =CH, benzamide $C_4$), 7.64 - 7.54 (m, 5H, benzamide $C_3$, $C_5$, phenyl $C_4$, $C_5$, dimethoxyphenyl $C_2$), 7.42 - 7.40 (dd, *J* = 8.5Hz, 2Hz, dimethoxyphenyl $C_5$), 7.05 (d, *J* = 8.5Hz, 1H dimethoxyphenyl $C_6$), 3.87 (s, 3H, -OMe $C_3$), 3.83 (s, 3H, -OMe $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.54 (-C=O, acryloyl), 166.20 (-C=O, amide), 151.82 (dimethoxyphenyl $C_3$), 149.50 (dimethoxyphenyl $C_4$), 145.21 (β =CH), 140.10 (phenyl $C_3$), 138.87 (phenyl $C_1$), 135.09 (benzamide $C_1$), 132.24 (phenyl $C_5$), 129.52 (benzamide $C_4$), 128.91(benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 127.93 (phenyl $C_6$), 125.17(dimethoxyphenyl $C_1$), 124.47 (phenyl $C_4$), 124.32 (dimethoxyphenyl $C_6$), 120.46 (α =CH), 120.23 (phenyl $C_2$), 112.08

(dimethoxyphenyl $C_5$), 111.36 (dimethoxyphenyl $C_2$), 56.22 (-OMe), 56.09 (-OMe); MS (ESI+): *m/z* calculated for $C_{24}H_{21}NO_4$: 387.44, found – 388.08 (M+ 1).

*N-(3-(3-(4-(trifluoromethyl)phenyl)acryloyl)phenyl)benzamide* **(34)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **14,** obtained as a white Solid, yield - 97%, M.P.- 144-145 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.50 (s, 1H, amide NH), 8.49 (s, 1H, phenyl $C_2$), 8.16 (d, *J* = 8Hz, 1H, phenyl $C_6$ ), 8.12 (d, *J* = 8Hz, 2H, benzamide $C_2$, $C_6$), 8.03 - 7.98 (m, 4H, benzamide $C_4$, β =CH, phenyl $C_4$, phenyl $C_5$), 7.84 - 7.81 (m, 3H, α =CH, (trifluoromethyl)phenyl $C_2$, $C_6$), 7.63 - 7.54 (m, 4H, (trifluoromethyl)phenyl $C_3$, $C_5$, benzamide $C_3$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.53 (-C=O, acryloyl), 166.23 (-C=O, amide), 142.56 (β =CH), 140.21 (phenyl $C_3$), 139.13 ((trifluoromethyl)phenyl $C_1$), 138.20 (phenyl $C_1$), 135.05 (benzamide $C_1$), 132.25 (phenyl $C_5$), 130.70 (benzamide $C_4$), 130.45 ((trifluoromethyl)phenyl $C_4$), 129.86 (-$CF_3$,), 129.67 (phenyl $C_6$), 128.91 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 126.21 ((trifluoromethyl)phenyl $C_2$), 126.18 ((trifluoromethyl)phenyl $C_6$), 125.62 ((trifluoromethyl)phenyl $C_5$), 125.26 ((trifluoromethyl)phenyl $C_3$), 124.67 (phenyl $C_4$), 123.43 (α =CH), 120.60 (phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3NO_2$: 395.38, found – 396.2 (M+1).

*N-(3-(3-(3-(trifluoromethyl)phenyl)acryloyl)phenyl)benzamide* **(35)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **15,** obtained as a white solid, yield - 96%, M.P.- 141-142 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.50 (s, 1H, amide NH), 8.48 (s, 1H, phenyl $C_2$), 8.32 (s, 1H, phenyl $C_6$), 8.19 - 8.15 (dd, *J* = 14Hz, 8Hz, 2H, benzamide $C_2$, $C_6$), 8.08 (d, *J* = 16Hz, 1H, β =CH), 8.02 (d, *J* = 7.5Hz, 3H, benzamide $C_3$, $C_5$, phenyl $C_5$), 7.87 (d, *J* = 16Hz, 1H, α =CH), 7.81 (d, *J* = 7.5Hz, 1H, phenyl $C_4$), 7.71 (t, *J* = 8Hz, 1H,

(trifluoromethyl)phenyl) $C_5$), 7.63 - 7.54 (m, 4H, benzamide $C_4$, (trifluoromethyl) phenyl) $C_2$, C4, C6). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.55 (-C=O, acryloyl), 166.23 (-C=O, amide), 142.75 (β =CH), 140.18 (phenyl $C_3$), 138.27 (phenyl $C_1$), 136.30 ((trifluoromethyl)phenyl) $C_1$), 135.06 (benzamide $C_1$), 133.21 (phenyl $C_5$), 132.25 (benzamide $C_4$), 130.44 ((trifluoromethyl)phenyl) $C_6$), 130.21 ((trifluoromethyl)phenyl) $C_3$), 129.62 (phenyl $C_6$), 128.91 (benzamide $C_3$, $C_5$, (trifluoromethyl)phenyl) $C_2$), 128.18 (benzamide $C_2$, $C_6$), 127.19 (-$CF_3$), 125.63 ((trifluoromethyl)phenyl) $C_4$), 124.78 (phenyl $C_4$), 124.57 ((trifluoromethyl)phenyl) $C_2$), 123.44 (α =CH), 120.60 (phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3NO_2$: 395.38, found – 396.5 (M+1).

*N-(3-(3-(4-(trifluoromethoxy)phenyl)acryloyl)phenyl)benzamide* **(36)**: This compound also was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **16,** obtained as a white solid, yield - 92%, M.P.- 136-137 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.48 – 8.47 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.15 - 8.14 (dd, *J* = 1, 1.5 Hz, 1H, phenyl $C_6$), 8.06 - 8.00 (m, 4H, benzamide $C_2$, $C_6$, phenyl $C_4$, phenyl $C_5$), 7.98 - 7.96 (d, *J* = 8Hz, 1H, benzamide $C_4$), 7.94 - 7.91 (d, *J* = 16Hz, 1H, β =CH), 7.81 - 7.78 (d, *J* = 15.5Hz, 1H, α =CH), 7.64 - 7.54 (m, 4H, (trifluoromethoxy)phenyl $C_2$, $C_3$, $C_5$, $C_6$), 7.48 - 7.46 (d, *J* = 8Hz, 2H, benzamide $C_3$, $C_5$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 194.28 (-C=O, acryloyl), 170.95 (-C=O, amide), 154.83 ((trifluoromethoxy)phenyl $C_4$), 147.56 (β =CH), 144.96 ((trifluoromethoxy)phenyl $C_3$), 143.10 ((trifluoromethoxy)phenyl $C_5$), 139.82 (phenyl $C_3$), 139.22 (phenyl $C_1$), 137.00 (benzamide $C_4$), 136.06 (benzamide $C_1$), 134.37 (phenyl $C_5$), 133.66 ((trifluoromethoxy)phenyl $C_2$), 132.94 ((trifluoromethoxy)phenyl $C_6$), 130.27 (-$OCF_3$), 129.34 (phenyl $C_6$), 128.57 (α =CH), 126.59 (benzamide $C_5$), 126.52 (benzamide $C_3$), 126.25 (phenyl $C_2$), 125.36 (benzamide $C_2$), 125.25 (benzamide

$C_6$), 124.21 (phenyl $C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3NO_3$: 411.38, found – 412.2 (M+1).

*N-(3-(3-(4-cyanophenyl)acryloyl)phenyl)benzamide* **(37)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **17,** obtained as an off white solid, yield - 92%, M.P.- 122-123 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.48 (s, 1H, amide NH), 8.47 (t, *J* = 1.5Hz, 1H, phenyl $C_2$), 8.15 - 8.13 (dd, *J* = 8Hz, 1.5Hz, 1H, phenyl $C_6$), 8.09 (d, *J* = 8Hz, 2H, benzamide $C_2$, $C_6$), 8.04 - 7.97 (m, 4H, β =CH, benzamide $C_4$, cyanophenyl $C_3$, $C_5$), 7.94 (d, *J* = 8.5Hz, 2H, benzamide $C_3$, $C_5$), 7.81 (d, *J* = 16Hz, 1H, α =CH), 7.63 - 7.53 (m, 4H, phenyl $C_4$, $C_5$, cyanophenyl $C_2$, $C_6$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.03 (C=O, acryloyl), 165.74 (-C=O, amide), 141.83 (β =CH), 139.73 (cyanophenyl $C_1$), 139.20 (phenyl $C_3$), 137.68 (phenyl $C_1$), 134.56 (benzamide $C_1$), 132.73 (cyanophenyl $C_3$,$C_5$), 131.76 (phenyl $C_5$), 129.38 (benzamide $C_3$, $C_5$), 129.19 (benzamide $C_4$), 128.42 (cyanophenyl $C_2$,$C_6$), 127.69 (benzamide $C_2$, $C_6$), 125.43 (phenyl $C_6$), 125.20 (phenyl $C_4$), 124.22(α =CH), 120.14 (phenyl $C_2$), 118.60 (-CN), 112.35 (cyanophenyl $C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}N_2O_2$: 352.39, found – 353.0 (M+1).

*N-(3-(3-(3-cyanophenyl)acryloyl)phenyl)benzamide* **(38)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **18,** obtained as an off white solid, yield - 93%, M.P.- 115-116 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.48 (t, *J* = 2Hz, 2H, phenyl $C_2$, cyanophenyl $C_2$), 8.20 (d, *J* = 8Hz, 1H, cyanophenyl $C_4$), 8.16 - 8.14 (dd, *J* = 8Hz, 1Hz, 1H, phenyl $C_6$), 8.07 (d, *J* = 16Hz, 1H, β =CH), 8.02 - 8.01 (m, 3H, benzamide $C_2$, $C_6$, cyanophenyl $C_6$), 7.91 - 7.89 (dt, 1H, benzamide $C_4$), 7.80 (d, *J* = 16Hz, 1H, α =CH), 7.69 (t, *J* = 8Hz, 1H, phenyl $C_5$), 7.64 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, cyanophenyl $C_5$, phenyl $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.43 (-C=O,

acryloyl), 166.21 (-C=O, amide), 142.09 (β =CH), 140.21 (phenyl $C_3$), 138.23 (phenyl $C_1$), 136.45 (cyanophenyl $C_1$), 135.05 (benzamide $C_1$), 134.04 (phenyl $C_5$), 134.00 (cyanophenyl $C_6$), 132.41 (benzamide $C_4$), 132.25 (cyanophenyl $C_4$), 130.60 (cyanophenyl $C_2$), 129.63 (cyanophenyl $C_5$), 128.91 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 125.70 (phenyl $C_6$), 124.89 (phenyl $C_4$), 124.75 (α =CH), 120.62 (phenyl $C_2$), 118.93 (-CN), 112.65 (cyanophenyl $C_3$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}N_2O_2$: 352.39, found – 353.5 (M+1).

*N-(3-(3-(p-tolyl)acryloyl)phenyl)benzamide* **(39)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **19,** obtained as a white solid, yield - 89%, M.P.- 118-119 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.47 (s, 1H, amide NH), 8.46 (t, *J* = 2Hz, 1H, phenyl $C_2$), 8.15 - 8.13 (dd, *J* = 8Hz, 1.5 Hz, 1H, phenyl $C_6$), 8.02 (d, *J* = 7Hz, 2H, benzamide $C_2$, $C_6$), 7.94 (d, *J* = 7.5Hz, 1H,benzamide $C_4$), 7.84 - 7.74 (m, 4H, β =CH, phenyl C4, $C_5$, α =CH), 7.63 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, *p*-tolyl $C_2$, $C_6$ ), 7.30 (d, *J* = 8Hz, 2H, *p*-tolyl $C_3$, $C_5$), 2.36 (-$CH_3$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.57 (-C=O, acryloyl), 166.21 (-C=O, amide), 144.71 (β =CH), 141.28 (phenyl $C_3$), 140.15 (*p*-tolyl $C_4$), 138.64 (phenyl $C_1$), 135.10 (benzamide $C_1$), 132.39 (phenyl $C_5$), 132.23 (*p*-tolyl $C_1$), 130.07 (benzamide $C_3$, $C_5$), 129.60 (benzamide $C_4$), 129.33 (*p*-tolyl $C_3$, $C_5$), 128.91 (*p*-tolyl $C_2$, $C_6$), 128.18 (benzamide $C_2$, $C_6$), 125.26 (phenyl $C_6$), 124.40 (phenyl $C_4$), 121.55 (α =CH), 120.54 (phenyl $C_2$), 21.57 (-$CH_3$); MS (ESI+): *m/z* calculated for $C_{23}H_{19}NO_2$: 341.41, found – 342.5 (M+1).

*N-(3-(3-(o-tolyl)acryloyl)phenyl)benzamide* **(40)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **20,** obtained as a white solid, yield - 91%, M.P.- 132-133 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.48 (s, 1H, amide NH), 8.48 (t, *J* = 2Hz, phenyl $C_2$), 8.16 - 8.14 (dd, *J* = 8Hz,

1Hz, 1H, phenyl $C_6$), 8.03 - 8.00 (m, 3H, benzamide $C_2$, $C_6$, β =CH), 7.97 (d, *J* = 7.5Hz, benzamide $C_4$), 7.94 (d, *J* = 8Hz, phenyl $C_5$), 7.76 (d, *J* = 15.5Hz, α =CH), 7.64 - 7.54 (m, 4H, *o*-tolyl $C_5$, $C_6$, benzamide $C_3$, $C_5$), 7.38 - 7.35 (m, 1H, phenyl $C_4$), 7.32 - 7.30 (m, 2H, *o*-tolyl $C_3$, $C_4$), 2.46 (s, 3H, -$CH_3$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.66 (-C=O, acryloyl), 166.24 (-C=O, amide), 141.73 (β =CH), 140.18 (phenyl $C_3$), 138.52 (phenyl $C_1$), 135.11 (benzamide $C_1$), 133.76 (*o*-tolyl $C_1$), 132.23 (*o*-tolyl $C_2$), 131.32 (phenyl $C_5$), 130.92 (benzamide $C_4$), 129.65 (*o*-tolyl $C_4$, $C_6$), 128.91 (benzamide $C_3$, $C_5$), 128.18 (benzamide $C_2$, $C_6$), 127.25 (phenyl $C_6$), 126.90 (*o*-tolyl $C_3$), 125.31 (*o*-tolyl $C_5$), 124.42 (phenyl $C_4$), 123.58 (α =CH), 120.60 (phenyl $C_2$), 19.82 (-$CH_3$); MS (ESI+): *m/z* calculated for $C_{23}H_{19}NO_2$: 341.41,found – 342.5 (M+1).

*N-(3-(3-(4-isopropylphenyl)acryloyl)phenyl)benzamide* **(41)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **21,** obtained as a pale yellow solid, yield - 98%, M.P.- 134-135 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 10.49 (s, 1H, amide NH), 8.47 (s, 1H, phenyl $C_2$), 8.15 (d, *J* = 7.5Hz, 1H, phenyl $C_6$), 8.02 (d, *J* = 7.5Hz, 2H, benzamide $C_2$, $C_6$), 7.94 (d, *J* = 8Hz, 1H, β =CH), 7.83 - 7.74 (m, 4H, benzamide $C_3$, $C_4$, $C_5$, phenyl $C_4$), 7.63 - 7.54 (m, 4H, Phenyl $C_5$, α =CH, , isopropylphenyl $C_2$, $C_6$), 7.34 (d, *J* = 7.5Hz, isopropylphenyl $C_3$, $C_5$), 2.95 - 2.90 (m, 1H, isopropyl CH), 1.22 (d, *J* = 6.5Hz, 6H, isopropyl -$CH_3$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 189.61 (-C=O, acryloyl), 166.22 (-C=O, amide), 152.03 (isopropylphenyl $C_4$), 144.73 (β =CH), 140.15 (phenyl $C_3$), 138.63 (phenyl $C_1$), 135.08 (benzamide $C_1$), 132.79 (phenyl $C_5$), 132.24 (benzamide $C_4$), 129.61 (isopropylphenyl $C_1$), 129.46 (benzamide $C_3$, $C_5$), 128.91 (isopropylphenyl $C_2$, $C_6$), 128.18 (benzamide $C_2$, $C_6$), 127.43 (isopropylphenyl $C_3$, $C_5$), 125.26 (phenyl $C_6$), 124.40 (phenyl $C_4$), 121.65 (α =CH), 120.53 (phenyl $C_2$), 33.90 (isopropyl CH), 24.07

(isopropyl -$CH_3$); MS (ESI+): *m/z* calculated for $C_{22}H_{23}NO_2$: 369.46, found –370.6 (M+1).

*N-(3-(3-(naphthalen-1-yl)acryloyl)phenyl)benzamide* **(42)**: This compound was synthesized as per general procedure described earlier in section 4.1.3.4 using compounds **2** and **22,** obtained as a white solid, yield - 95%, M.P.- 121-122 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 10.52 (s, 1H, amide NH), 8.61 (d, *J* = 15.5Hz, 1H, β =CH), 8.55 (s, 1H, phenyl $C_2$), 8.33 (d, *J* = 8Hz, 1H, naphthalen-1-yl $C_4$), 8.24 (d, *J* = 7.5Hz, 1H, naphthalen-1-yl $C_5$), 8.19 (d, *J* = 8Hz, 1H, naphthalen-1-yl $C_8$), 8.09 (d, *J* = 8Hz, 1H, naphthalen-1-yl $C_2$), 8.03 - 7.93 (m, 5H, benzamide $C_2$, $C_6$, phenyl $C_6$, $C_4$,α =CH, phenyl $C_6$), 7.68 - 7.55 (m, 7H, benzamide $C_3$, $C_4$, $C_5$, naphthalen-1-yl $C_7$, $C_3$, $C_6$, phenyl $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 189.53 (-C=O, acryloyl), 166.24 (-C=O, amide), 140.62 (phenyl $C_3$), 140.22 (phenyl $C_1$), 138.47 (β =CH), 135.10 (benzamide $C_1$), 133.86 (phenyl $C_5$), 132.25 (naphthalen-1-yl $C_1$), 131.86 (naphthalen-1-yl $C_{10}$), 131.67 (naphthalen-1-yl $C_9$), 131.39 (benzamide $C_4$), 129.70 (naphthalen-1-yl $C_5$), 129.28 (naphthalen-1-yl $C_4$), 128.92 (benzamide $C_3$, $C_5$), 128.20 (benzamide $C_2$, $C_6$), 127.77(phenyl $C_6$), 126.84 (naphthalen-1-yl $C_3$), 126.19 (naphthalen-1-yl $C_6$), 126.12 (naphthalen-1-yl $C_7$), 125.37 (naphthalen-1-yl $C_8$), 125.22 (naphthalen-1-yl $C_2$), 124.51 (phenyl $C_4$), 123.56 (α =CH), 120.65 (phenyl $C_2$); MS (ESI+): *m/z* calculated for $C_{26}H_{19}NO_2$: 377.44, found – 378.3 (M+1).

**4.1.3.5 General procedure for the synthesis of 3,5-diaryl-1H-pyrazole derivatives (43-62)**

To a stirred solution of compounds (**23-42**) in ethanol (10ml), hydrazine hydrate was added. The reaction mixture was refluxed for 2 h in oil bath and progress was monitored by TLC. After the completion of the reaction, solvent was evaporated and dried under reduced pressure. To this, DMSO and catalytic amount of iodine was added and heated

up to 110 °C for 1h. Then, the reaction mixture was cooled to room temperature and ice cold saturated sodium thiosulphate solution was added to afford solid precipitate. The formed precipitate was filtered, washed with cold water and dried under vacuum pump to get corresponding products **43**-**62**.

*N-(3-(3-phenyl-1H-pyrazol-5-yl)phenyl)benzamide* (**43**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **23** and obtained as an pale brown solid, yield- 84%, M.P.- 194-195 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.39 (s, 1H, pyrazole NH), 10.36 (s, 1H, amide NH), 8.30 (s, 1H, phenyl $C_2$), 8.01 (d, *J* = 10 Hz, 2H, phenyl $C_2$, $C_6$), 7.85 (s, 2H, benzamide $C_2$, $C_6$), 7.75 (s, 1H,benzamide $C_4$), 7.62-7.55 (m, 4H, benzamide $C_3$, $C_5$, phenyl $C_3$, $C_5$) 7.47 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.36 (s, 1H, phenyl $C_4$), 7.12 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 164.34 (-C=O, amide), 141.52 (pyrazole $C_3$, $C_5$), 135.24 (phenyl$_{(\text{pyrazole C5})}C_1$), 132.05 (benzamide $C_1$,$C_4$), 131.52 (phenyl$_{(\text{pyrazole C3})}$ $C_1$, phenyl$_{(\text{pyrazole C5})}$ $C_3$), 129.27 (phenyl $_{(\text{pyrazole C5})}$ $C_5$), 128.87 (benzamide $C_3$, $C_5$, phenyl$_{(\text{pyrazole C3})}$ $C_4$), 128.57 (phenyl$_{(\text{pyrazole C3})}$ $C_3$,$C_6$), 128.04 (benzamide $C_2$, $C_6$, phenyl$_{(\text{pyrazole C3})}C_2$, $C_6$), 121.82 (phenyl$_{(\text{pyrazole C5})}$ $C_4$, $C_6$), 117.98 (phenyl $_{(\text{pyrazole C5})}$ $C_2$), 100.12 (pyrazole $C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{17}N_3O$: 339.40, found 340.45 (M+1).

*N-(3-(3-(4-chlorophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**44**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **24** and obtained as a white solid, yield- 90%, M.P.- 236-237 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.46 (s, 1H, pyrazole NH), 10.37 (s, 1H, amide NH), 8.33 - 8.25 (d, *J* = 41Hz, 1H, phenyl $C_2$), 8.01 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.88 (bs, 2H, chlorophenyl $C_2$, $C_6$), 7.73 (bs, 1H, chlorophenyl $C_3$), 7.63 - 7.52 (m, 7H, benzamide $C_3$, $C_4$, & $C_5$ phenyl $C_4$, $C_5$, $C_6$, chlorophenyl $C_5$), 7.16 (s, 1H, pyrazole $C_4$).

$^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.06 (-C=O, amide), 140.11 (pyrazole $C_3$, $C_5$), 135.29 (phenyl $C_1$), 132.12 (benzamide $C_1$), 129.50 (phenyl $C_3$), 128.90 (benzamide $C_3$, $C_5$, phenyl $C_5$), 128.15 (benzamide $C_4$, chlorophenyl $C_1$, $C_3$, $C_5$), 127.30 (benzamide $C_2$, $C_6$, chlorophenyl $C_2$, $C_6$), 121.18 (chlorophenyl $C_4$, phenyl $C_4$), 118.31 (phenyl $C_6$), 117.73 (phenyl $C_2$), 100.37 (pyrazole $C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}ClN_3O$: 373.84, found –374.0 (M+), 375.9 (M+2).

*N-(3-(3-(2-chlorophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**45**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **25** and obtained as an off white Solid, yield- 70%, M.P.- 191-192 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.47 (d, *J* = 125.0 Hz, 1H, pyrazole NH), 10.36 (s, 1H, amide NH), 8.26 (s, 1H, phenyl $C_2$), 8.01 (d, *J* = 10 Hz, 2H, benzamide $C_2$, $C_6$), 7.81 (d, *J* = 10 Hz, 2H, benzamide $C_3$, $C_5$), 7.63 - 7.54 (m, 5H, chlorophenyl $C_6$ phenyl $C_4$, $C_5$, $C_6$, benzamide $C_4$, ), 7.45 (m, 3H, chlorophenyl $C_3$, $C_4$, $C_5$), 7.08 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (126 MHz, DMSO) $\delta_C$ 166.10 (-C=O, amide), 140.12 (pyrazole $C_3$, $C_5$), 135.32 (phenyl $C_1$), 132.11 (benzamide $C_1$), 131.48 (phenyl $C_3$), 130.86 (chlorophenyl $C_2$, $C_3$), 129.61 (chlorophenyl $C_1$), 128.88 (benzamide $C_3$, $C_5$, C4, chlorophenyl $C_6$), 128.15 (benzamide $C_2$, $C_6$, chlorophenyl $C_4$, $C_5$), 127.92 (phenyl $C_5$), 121.08 (phenyl $C_4$, $C_6$), 117.92 (phenyl $C_2$), 103.64 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}ClN_3O$: 373.84, found – 374.1067 (M+), 376.1049 (M+2).

*N-(3-(3-(2,4-dichlorophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**46**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **26** and obtained as an off white solid, yield- 78%, M.P.- 195-196 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.69 (s, 1H, pyrazole NH), 10.36 (s, 1H, amide NH), 8.23 (s, 1H, phenyl $C_2$), 8.01-7.99 (m, 2H, benzamide $C_2$, $C_6$), 7.91- 7.75 (m, 3H, phenyl $C_4$, C6, 2,4-dichlorophenyl $C_6$), 7.63 - 7.46 (m, 6H, benzamide $C_3$, $C_4$, $C_5$, 2,4-

dichlorophenyl $C_3$, $C_5$, phenyl $C_5$), 7.10 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.12 (-C=O, amide), 140.14 (pyrazole $C_3$, $C_5$), 135.27 (phenyl $C_1$), 132.33 (2,4-dichlorophenyl $C_4$), 132.14 (benzamide $C_1$, 2,4-dichlorophenyl $C_2$ ), 131.98 (benzamide $C_4$, phenyl $C_3$), 130.21 (2,4-dichlorophenyl $C_6$), 128.89 (benzamide $C_3$, $C_5$, 2,4-dichlorophenyl $C_3$, phenyl $C_5$), 128.13 (benzamide $C_2$, $C_6$, 2,4-dichlorophenyl $C_1$, $C_5$), 121.10 (phenyl $C_4$, $C_6$ ) , 118.04 (phenyl $C_2$), 103.70 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{15}Cl_2N_3O$: 408.28, found - 408.0 (M+), 409.9 (M+2).

*N-(3-(3-(4-bromophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**47**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **27** and obtained as a pale brown solid, yield- 84%, M.P.- 261-261 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.46 (s, 1H, pyrazole NH), 10.38 (s, 1H, amide NH), 8.34 (s, 1H, phenyl $C_2$), 8.02 (d, $J$ = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.84 (m, 2H, bromophenyl $C_2$, $C_6$), 7.69 - 7.54 (m, 7H, benzamide $C_3$, $C_4$, & $C_5$ phenyl $C_4$, $C_5$, $C_6$ & bromophenyl $C_5$), 7.46 (m, 1H, bromophenyl $C_3$), 7.17 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.07 (-C=O, amide), 140.02 (pyrazole $C_3$, $C_5$), 135.29 (phenyl $C_1$), 132.11 (benzamide $C_1$), 129.82 (phenyl $C_3$), 128.90 (benzamide $C_3$, $C_5$ & phenyl $C_5$), 128.14 (benzamide $C_4$ & bromophenyl $C_1$, $C_3$, $C_5$), 127.60 (benzamide $C_2$, $C_6$ & bromophenyl $C_2$, $C_6$), 121.18 (bromophenyl $C_4$ & phenyl $C_4$), 118.33 (phenyl $C_6$), 117.74 (phenyl $C_2$), 100.37 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}BrN_3O$: 418.29, found - 417.9 (M+), 419.9 (M+2).

*N-(3-(3-(3-bromophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**48**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **28** and obtained as a pale brown solid, yield- 88%, M.P.- 249-250 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.51 (d, $J$ = 16.5Hz, 1H, pyrazole NH), 10.38 (d, $J$ = 22.5Hz, 1H, amide NH), 8.35 (d, 1H, $J$ = 50 Hz, phenyl $C_2$), 8.09 (s, 1H, bromophenyl

$C_2$), 8.02 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.89 (d, *J* = 17 Hz, 1H, bromophenyl $C_6$), 7.76 (d, *J* = 23.5Hz, 1H, bromophenyl $C_4$), 7.63 - 7.43 (m, 7H, benzamide $C_3$, $C_4$, & $C_5$ phenyl $C_4$, $C_5$, $C_6$ & bromophenyl $C_5$), 7.25 (d, *J* = 26.5 Hz, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.06 (-C=O, amide), 140.12 (pyrazole $C_3$, $C_5$) 135.30 (phenyl $C_1$), 132.13 (benzamide $C_1$), 131.38 (phenyl $C_3$), 128.90 (benzamide $C_3$, $C_5$ & bromophenyl $C_1$), 128.14 (benzamide $C_4$ & bromophenyl $C_2$, $C_4$, $C_5$ & phenyl $C_5$), 128.05 (benzamide $C_2$, $C_6$ & bromophenyl $C_6$), 124.55 (bromophenyl $C_3$, phenyl $C_4$ ), 121.22 (phenyl $C_6$), 118.38 (phenyl $C_2$), 100.93 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}BrN_3O$: 418.29, found - 417.8 (M+), 419.9 (M+2).

*N-(3-(3-(4-fluorophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**49**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **29** and obtained as a white solid, yield- 79%, M.P.- 192-193 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.37 (s, 1H, pyrazole NH), 10.35 (s, 1H, amide NH), 8.31 (m, 1H, phenyl $C_2$), 8.02 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.90 (bs, 2H, fluorophenyl $C_2$, $C_6$), 7.74 (bs, 1H, phenyl $C_5$), 7.63- 7.54 (m, 4H, benzamide $C_3$,$C_5$, phenyl $C_4$, $C_6$), 7.44 (bs, 1H, benzamide $C_4$), 7.30 (bs, 2H, fluorophenyl $C_3$, $C_5$), 7.10 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.07 (C=O, amide), 161.29 (fluorophenyl $C_4$), 140.04 (pyrazole $C_3$, $C_5$), 135.32 (phenyl $C_1$), 132.10 (benzamide $C_1$), 128.89 (benzamide $C_3$, $C_4$ $C_5$,), 128.14 (benzamide $C_2$, $C_6$,fluorophenyl $C_5$), 127.64 (fluorophenyl $C_1$), 121.19 (phenyl $C_4$), 116.36 (phenyl $C_2$), 100.09 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{22}H_{16}FN_3O$: 357.39, found – 356.3 (M+1).

*N-(3-(3-(3-fluorophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**50**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **30** and obtained as a white solid, yield- 75%, M.P.- 240-241 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.49 (s, 1H, pyrazole NH), 10.37 (s, 1H, amide NH), 8.30 (s,

1H, phenyl $C_2$), 8.03 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.74 - 7.69 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.63 - 7.46 (m, 6H, benzamide $C_4$, $C_3$, $C_5$, fluorophenyl $C_2$, $C_5$, $C_6$), 7.20 (bs, 2H, pyrazole $C_4$, fluorophenyl $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.09 (C=O, amide), 164.06 (fluorophenyl $C_3$), 162.13 (fluorophenyl $C_1$), 140.08 (pyrazole $C_3$, $C_5$), 135.31 (phenyl $C_1$), 132.11 (benzamide $C_1$, phenyl $C_3$), 129.57 (phenyl $C_5$), 128.89 (benzamide $C_3$, $C_4$ $C_5$,), 128.14 (benzamide $C_2$, $C_6$, fluorophenyl $C_5$), 121.68 (phenyl $C_4$, fluorophenyl $C_6$), 121.23 (phenyl $C_2$,$C_6$), 112.28 (fluorophenyl $C_2$), 112.11 (fluorophenyl $C_4$), 100.74 (pyrazole-$C_4$); MS (ESI+):*m/z* calculated for $C_{22}H_{16}FN_3O$: 357.39, found – 356.3 (M+1).

*N-(3-(3-(4-methoxyphenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**51**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **31** and obtained as a pale yellow, yield- 89%, M.P.- 207-208 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.20 (s, 1H, pyrazole NH), 10.34 (s, 1H, amide NH), 8.29 (s, 1H, phenyl $C_2$), 8.01 (d, *J* = 8.5 Hz, 2H, benzamide $C_2$, $C_6$), 7.79 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.63-7.54 (m, 4H, benzamide $C_3$, $C_5$ & methoxyphenyl $C_2$, $C_6$), 7.44 (t, 1H, J = 7.5 Hz, benzamide $C_4$), 7.04 (d, *J* = 9 Hz, 2H, methoxy phenyl $C_3$, $C_5$ ), 7.01 (s, 1H, pyrazole$C_4$), 3.81 (s, 3H, -OMe). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.03 (-C=O, amide), 160.86 (methoxyphenyl $C_4$), 140.00 (pyrazole $C_3$, $C_5$), 135.35 (phenyl $C_1$), 132.08 (benzamide $C_1$), 128.87 (benzamide $C_3$, $C_5$ & methoxyphenyl $C_2$, $C_6$), 128.14 (benzamide $C_2$, $C_6$), 126.97 (methoxyphenyl $C_1$ & phenyl $C_4$, $C_6$), 121.15 (phenyl $C_2$), 114.76 (methoxyphenyl $C_3$, $C_5$), 99.28 (pyrazole-$C_4$), 55.66 (-OMe); HRMS (ESI+): *m/z* calculated for $C_{23}H_{19}N_3O_2$: 369.42, found 370.1552 (M+1), 371.1604 (M+2).

*N-(3-(3-(3-methoxyphenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**52**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **32** and obtained as a pale yellow solid, yield- 93%, M.P.- 215-216 °C, $^1$H

NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.38 (s, 1H, pyrazole NH), 10.35 (s, 1H, amide NH), 8.32 (s, 1H, phenyl $C_2$), 8.02 (d, $J$ = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.76 (s, 1H, methoxyphenyl $C_2$), 7.63 - 7.55 (m, 4H, phenyl $C_4$, $C_5$, $C_6$ & benzamide $C_4$), 7.43 (m, 4H, benzamide $C_3$, $C_5$ & methoxyphenyl $C_5$, $C_6$ ), 7.15 (s, 1H, methoxyphenyl $C_4$), 6.93 (s, 1H, pyrazole $C_4$), 3.84 (s, 3H, -OMe). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.04 (-C=O, amide), 160.14 (methoxyphenyl $C_3$), 140.00 (pyrazole $C_3$, $C_5$), 135.34 (phenyl $C_1$), 132.09 (benzamide $C_1$), 128.88 (benzamide $C_3$, $C_5$ & methoxyphenyl $C_5$), 128.14 (Phenyl $C_4$, $C_6$ & benzamide $C_2$, $C_6$), 121.19 (Phenyl $C_2$ & methoxyphenyl $C_6$), 117.97 (methoxyphenyl $C_4$), 110.89 (methoxyphenyl $C_2$), 100.33 (pyrazole-$C_4$), 55.65 (-OMe); MS (ESI+): *m/z* calculated for $C_{23}H_{19}N_3O_2$: 369.42, found- 370.15 (M+1).

*N-(3-(3-(3,4-dimethoxyphenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**53**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **33** and obtained as a pale yellow solid, yield- 70%, M.P.- 105-106 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.22 (s, 1H, pyrazole NH), 10.33 (s, 1H, amide NH), 8.30 (s, 1H, phenyl $C_2$), 8.02 (d, $J$ = 7.5 Hz, 2H, benzamide $C_2$, $C_6$), 7.75 (s, 1H, phenyl $C_5$), 7.63 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, phenyl $C_4$, $C_6$), 7.44 - 7.38 (m, 3H, benzamide $C_4$, 3,4-dimethoxyphenyl $C_2$, $C_6$), 7.06 (s, 2H, pyrazole $C_4$, 3,4-dimethoxy phenyl $C_5$), 3.86 (s, 3H, methoxyphenyl $C_3$ -Me), 3.80 (s, 3H, methoxyphenyl $C_4$ -Me). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.04 (-C=O, amide), 149.51 (3,4-dimethoxyphenyl $C_3$, $C_4$), 139.98 (pyrazole $C_3$, $C_5$), 135.34 (phenyl $C_1$), 132.09 (benzamide C1, phenyl $C_3$), 128.88 (benzamide $C_3$, $C_5$, phenyl $C_5$), 128.13 (benzamide $C_2$, $C_6$, 3,4-dimethoxyphenyl $C_1$), 121.17 (phenyl $C_4$, $C_6$), 118.10 (phenyl $C_2$, 3,4-dimethoxyphenyl $C_6$), 112.60 (3,4-dimethoxyphenyl $C_5$), 109.54 (3,4-dimethoxyphenyl $C_2$) , 99.45 (pyrazole $C_4$), 56.07 (-OMe); MS (ESI+): *m/z* calculated for $C_{24}H_{21}N_3O_3$: 399.45 found – 400.0 (M+1).

*N-(3-(3-(4-(trifluoromethyl)phenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**54**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **34** and obtained as an off white solid, yield- 83%, M.P.- 210-211 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.91 (d, *J* = 149 Hz, 1H, pyrazole NH), 10.46 (d, *J* = 41.5 Hz, 1H, amide NH), 8.31 (s, 1H, phenyl $C_2$), 8.09 - 7.99 (m, 4H, benzamide $C_2$, $C_6$ (trifluoromethyl)phenyl) $C_3$, $C_5$), 7.88 - 7.74 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.63 - 7.47 (m, 5H, benzamide $C_4$, (trifluoromethyl)phenyl) $C_2$, $C_6$, benzamide $C_4$, $C_3$, $C_5$), 7.27 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.11 (C=O, amide), 140.10 (pyrazole $C_3$, $C_5$), 135.25 (phenyl $C_1$, (trifluoromethyl)phenyl $C_1$), 132.16 (benzamide $C_1$, phenyl $C_3$), 128.92 ((trifluoromethyl)phenyl $C_2$, $C_6$, benzamide $C_4$), 128.18 (phenyl $C_5$), 128.14 (benzamide $C_3$, $C_5$,), 126.26 (-$CF_3$), 126.12 ((trifluoromethyl)phenyl $C_3$,$C_5$, benzamide $C_2$, $C_6$), 121.22 (phenyl $C_6$, $C_4$), 120.63 (phenyl $C_2$), 101.14 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3N_3O$: 407.40, found – 408.52 (M+1).

*N-(3-(3-(3-(trifluoromethyl)phenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**55**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **35** and obtained as an off white solid, yield- 81%, M.P.- 215-217 °C, $^{1}$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.89 - 13.57 (d, *J* = 160Hz, pyrazole NH), 10.50 – 10.39 (d, *J* = 53.5 Hz, amide NH), 8.34 - 8.16 (m, 3H, (trifluoromethyl)phenyl $C_2$, $C_6$ phenyl $C_2$), 8.02 - 7.98 (m, 2H, benzamide $C_2$, $C_6$), 7.88 - 7.42 (m, 8H, benzamide $C_4$, $C_3$, $C_5$ phenyl $C_4$, $C_5$, $C_6$, (trifluoromethyl)phenyl $C_4$, $C_5$), 7.31(s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.07 (C=O, amide), 140.09 (pyrazole $C_3$, $C_5$), 135.28 (phenyl $C_1$), 132.16 (benzamide $C_1$), 130.05 (benzamide $C_4$) 129.43 ((trifluoromethyl) phenyl $C_1$, phenyl $C_3$), 128.91 (benzamide $C_3$, $C_5$, (trifluoromethyl) phenyl $C_3$,$C_5$), 128.18 (phenyl $C_5$, (trifluoromethyl)phenyl $C_6$), 128.14 (benzamide $C_2$, $C_6$, (trifluoromethyl) phenyl $C_2$, $C_4$), 121.89 (phenyl $C_6$), 121.22 (phenyl $C_4$), 120.64

(phenyl $C_2$), 100.86 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3N_3O$: 407.40, found – 408.53 (M+1).

*N-(3-(3-(4-(trifluoromethoxy)phenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**56**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **36** and obtained as a white solid, yield- 84%, M.P.- 210-211 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.51 (s, 1H, pyrazole NH), 10.41 (d, *J* = 24.5 Hz, 1H, amide NH), 8.36 (d, *J* = 49.5 Hz, 1H, phenyl $C_2$), 8.02 - 7.97 (m, 4H, benzamide $C_2$, $C_6$, (trifluoromethyl)phenyl) $C_3$, $C_5$), 7.77 (d, *J* = 19.5 Hz, 1H, phenyl $C_5$), 7.63 - 7.44 (m, 7H, benzamide $C_3$, $C_4$, $C_5$, (trifluoromethyl)phenyl) $C_2$, $C_6$, phenyl $C_4$, $C_6$), 7.19 (d, *J* = 20Hz, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.09 (C=O, amide), 150.48 ((trifluoromethyl)phenyl) $C_4$), 140.16 (pyrazole $C_3$, $C_5$), 135.24 (phenyl $C_1$), 132.12 (benzamide $C_1$, phenyl $C_3$), 129.79 (phenyl $C_5$), 128.89 (benzamide $C_3$, $C_4$, $C_5$), 128.15 ((trifluoromethyl)phenyl) $C_2$, $C_6$ benzamide $C_2$, $C_6$), 127.39 ((trifluoromethyl) phenyl) $C_3$, $C_5$), 122.14 ((trifluoromethyl)phenyl) $C_1$), 121.18 (phenyl $C_6$, $C_4$), 119.57 (phenyl $C_2$), 100.40 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}F_3N_3O_2$: 423.40, found – 424.1 (M+1).

*N-(3-(3-(4-cyanophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**57**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **37** and obtained as a pale yellow solid, yield- 79%, M.P.- 225-226 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.67 (s, 1H, pyrazole NH), 10.41 (s, 1H, amide NH), 8.37 (d, *J* = 51.5 Hz, 1H, phenyl $C_2$), 8.09 - 7.90 (m, 6H, benzamide $C_2$, $C_6$, cyanophenyl $C_2$, $C_3$, $C_5$, $C_6$), 7.77 (m, 1H,benzamide $C_4$ ), 7.63 - 7.44 (m, 5H, phenyl $C_4$, $C_5$, $C_6$ benzamide $C_3$, $C_5$), 7.35 (d, *J* = 41.5 Hz, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$ 166.11 (-C=O, amide), 144.50 (pyrazole $C_3$), 140.20 (pyrazole $C_5$), 138.51 (cyanophenyl $C_1$), 135.22 (phenyl $C_1$), 133.53 (benzamide $C_1$), 133.22 (phenyl

$C_3$), 132.15 (benzamide $C_4$), 129.82 (phenyl $C_5$), 128.91 (phenyl C4, cyanophenyl $C_3$, $C_5$), 128.14 (benzamide $C_3$, $C_5$, $C_2$, $C_6$), 126.17 (phenyl $C_6$, cyanophenyl $C_2$, $C_6$), 121.23 (phenyl $C_2$), 118.37 (-CN), 110.19 (cyanophenyl $C_4$), 101.19 (pyrazole-$C_4$); HRMS (ESI+): *m/z* calculated for $C_{23}H_{16}N_4O$: 364.41, found - 365.1405 (M+1), 366.1430 (M+2).

*N-(3-(3-(3-cyanophenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**58**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **38** and obtained as a yellow solid, yield- 85%, M.P.- 215-216 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) δ 13.53 (s, 1H, pyrazole NH), 10.43 (s, 1H, amide NH), 8.32 (s, 1H, phenyl $C_2$), 8.22 (d, *J* = 7.5 Hz, 2H, benzamide $C_2$, $C_6$), 8.05 - 7.98 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.81 (d, *J* = 7 Hz, 1H, , cyanophenyl $C_4$), 7.73 - 7.43 (m, 7H, benzamide $C_4$, $C_3$, $C_5$, cyanophenyl $C_2$, $C_5$, $C_6$), 7.30 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 166.11 (-C=O, amide), 140.10 (pyrazole $C_3$, $C_5$), 135.26 (phenyl $C_1$), 132.20 (benzamide $C_1$), 131.70 (cyanophenyl $C_1$), 130.61 (benzamide $C_4$), 130.04 (cyanophenyl $C_4$), 129.66 (phenyl $C_3$), 129.34 (cyanophenyl $C_6$), 128.97 (cyanophenyl $C_2$), 128.91 (cyanophenyl $C_5$), 128.90 (phenyl $C_5$), 128.16 (benzamide $C_3$, $C_5$), 128.13 (benzamide $C_2$, $C_6$), 124.90 (phenyl $C_4$), 121.20 (phenyl $C_6$), 119.18 (phenyl $C_2$), 118.12 (-CN), 112.49 (cyanophenyl $C_3$), 100.99 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{23}H_{16}N_4O$ 364.41, found – 365.52 (M+1).

*N-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**59**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **39** and obtained as a pale yellow solid, yield- 70%, M.P.- 182-183 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 13.32 (s, 1H, pyrazole NH), 10.35 (s, 1H, amide NH), 8.32 (s, 1H, phenyl $C_2$), 8.02 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.74 (s, 3H, phenyl $C_4$,$C_5$ $C_6$,), 7.63 - 7.54 (m, 4H, tolyl $C_2$, $C_6$ & benzamide $C_3$, $C_5$), 7.43 (s, 1H,

benzamide $C_4$ ), 7.28 (s, 2H, tolyl $C_3$, $C_5$), 7.06 (s, 1H, pyrazole $C_4$), 2.34 (s, 3H, -Me). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.05 (-C=O, amide), 140.00 (pyrazole $C_3$, $C_5$), 135.35 (phenyl $C_1$), 132.09 (benzamide $C_1$), 129.93 (phenyl $C_3$), 128.88 (benzamide $C_3$, $C_5$, tolyl $C_1$,$C_4$), 128.14 (tolyl $C_3$, $C_5$, phenyl $C_5$, benzamide $C_4$), 125.53 (benzamide $C_2$ $C_6$, tolyl $C_2$, $C_6$), 121.18 (phenyl $C_4$, $C_6$), 117.69 (phenyl $C_2$), 99.70 (pyrazole-$C_4$), 21.30 (-Me); HRMS (ESI+): *m/z* calculated for $C_{23}H_{19}N_3O$:353.43, found - 354.1600 (M+1), 355.1632 (M+2).

*N-(3-(3-(o-tolyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**60**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **40** and obtained as an off white, yield- 75%, M.P.- 174-175 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.11 (s, 1H, pyrazole NH), 10.34 (s, 1H, amide NH), 8.27 (s, 1H, phenyl $C_2$), 8.01 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.79 (d, *J* = 8 Hz, 1H, tolyl $C_6$), 7.62 - 7.59 (m, 2H, phenyl $C_4$, $C_6$), 7.57 - 7.54 (m, 3H, phenyl $C_5$, tolyl $C_3$, $C_4$), 7.45 (t, *J* = 8 Hz, 1H, benzamide $C_4$ ), 7.31 (m, 3H, benzamide $C_3$, $C_5$ & tolyl $C_5$), 6.86 (s, 1H, pyrazole $C_4$), 2.47(s, 3H, -Me). $^{13}$C NMR (125 MHz, DMSO-*d*$_6$) $\delta_C$ 166.08 (-C=O, amide), 139.99 (pyrazole $C_3$, $C_5$), 135.84 (phenyl $C_1$), 135.35 (tolyl $C_2$), 132.09 (benzamide $C_1$), 131.31 (benzamide $C_4$, tolyl $C_1$), 129.19 (phenyl C3, tolyl $C_6$), 128.88 (benzamide $C_3$, $C_5$, tolyl $C_4$), 128.16 (tolyl $C_3$), 128.14 (benzamide $C_2$, $C_6$, tolyl $C_5$), 126.46 (Phenyl $C_5$), 121.22 (phenyl $C_4$), 120.39 (phenyl $C_6$), 117.90 (phenyl $C_2$), 102.89 (pyrazole $C_4$), 21.22 (-Me); MS (ESI+): *m/z* calculated for $C_{23}H_{19}N_3O$-353.43, found – 354.16 (M+1), 355.17 (M+2).

*N-(3-(3-(4-isopropylphenyl)-1H-pyrazol-5-yl)phenyl)benzamide* (**61**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **41** and obtained as an off white solid, yield- 73%, M.P.- 207-208 °C, $^1$H NMR (500 MHz, DMSO-*d*$_6$) $\delta_H$ 13.32 (s, 1H, pyrazole NH), 10.34 (s, 1H, amide NH),

8.31 (s, 1H, phenyl $C_2$), 8.03 (d, *J* = 7 Hz, 2H, benzamide $C_2$, $C_6$), 7.76 (bs, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.63 - 7.54 (m, 4H, benzamide $C_3$, $C_5$, isopropylphenyl $C_2$, $C_6$), 7.43 (bs, 1H, benzamide $C_4$), 7.34 (bs, 2H, isopropylphenyl $C_3$, $C_5$), 7.05 (s, 1H, pyrazole $C_4$), 2.95 - 2.90 (m, 1H –CH), 1.24 (s, 3H, -$CH_3$), 1.23 (s, 3H, -$CH_3$). $^{13}$C NMR (125 MHz, DMSO-*$d_6$*) $\delta_C$ 166.06 (C=O, amide), 140.01 (pyrazole $C_3$, $C_5$), 135.36 (phenyl $C_1$), 132.08 (benzamide $C_1$, $C_4$), 129.43 (phenyl $C_3$), 128.88 (benzamide $C_3$, $C_5$ phenyl $C_5$), 128.14 (isopropylphenyl $C_3$, $C_5$ benzamide $C_2$, $C_6$), 127.31 (isopropylphenyl $C_1$), 125.65 (isopropylphenyl $C_2$, $C_6$), 121.17 (phenyl $C_4$, $C_6$), 117.79 (phenyl $C_2$), 99.72 (pyrazole $C_4$), 33.68 (isopropyl -CH), 24.24 (isopropyl –$CH_3$); MS (ESI+): *m/z* calculated for $C_{25}H_{23}N_3O$: 381.48, found – 382.5 (M+1).

*N-(3-(3-(naphthalen-1-yl)-1H-pyrazol-5-yl)phenyl)benzamide* (**62**): This compound was synthesized as per general procedure described earlier in section 4.1.3.5 using compound **42** and obtained as a pale yellow solid, yield- 90%, M.P.- 141-142 °C, $^1$H NMR (500 MHz, DMSO-*$d_6$*) $\delta_H$ 13.60 (s, 1H, pyrazole NH), 10.37 (s, 1H, amide NH), 8.03 - 8.01 (m, 4H, phenyl $C_2$, naphthalene $C_2$ $C_4$, $C_5$), 7.80 - 7.46 (m, 12H, benzamide $C_2$, $C_3$, $C_4$, $C_5$, $C_6$, phenyl $C_4$, $C_5$, $C_6$, naphthalene $C_3$, $C_6$, $C_7$, $C_8$), 7.02 (s, 1H, pyrazole $C_4$). $^{13}$C NMR (125 MHz, DMSO-*$d_6$*) $\delta_C$ 166.09 (C=O, amide), 140.06 (pyrazole $C_3$, $C_5$), 135.35 (phenyl $C_1$), 133.98 (naphthalene $C_1$), 132.10 (benzamide $C_1$), 131.02 (benzamide $C_4$), 128.89 (benzamide $C_2$, $C_6$, $C_3$, $C_5$), 128.18 (naphthalene $C_2$), 128.15 (naphthalene C4, $C_{4a}$, $C_{8a}$, phenyl $C_5$), 127.43 (naphthalene $C_5$, $C_8$), 125.99 (naphthalene $C_3$, $C_6$, $C_7$), 121.29 (phenyl $C_4$, $C_6$), 120.47 (phenyl $C_2$), 103.69 (pyrazole-$C_4$); MS (ESI+): *m/z* calculated for $C_{26}H_{19}N_3O$: 389.46, found – 390.51 (M+1).

**4.1.3.6 General procedure for the synthesis of compounds 63, 64 and 65**

Compounds **63**, **64**, **65** were synthesized according to a previously described method and compared with proton NMR data [Jha and Jain 2016].

**4.1.3.7 General procedure for the synthesis of spiropyrazolines derivatives (66-85)**

To a stirred solution of chalcone derivatives (**23-42**, 0.5 eq) in 1,4-Dioxane (5ml), cyclohexanone tosylhydrazone (**65**, 1.0 eq) and dry $Cs_2CO_3$ (1.0 eq) was added. The reaction mixture was refluxed for 2 h in oil bath under nitrogen atmosphere and progress was monitored by TLC. After the completion of the reaction, it was cooled to room temperature, and water (10 mL) and ethyl acetate (20 mL) were added. The two layers were separated, and the aqueous phase was extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with brine solution, dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel (60–120 mesh) to afford the desired spiropyrazoline **(66-85)**.

*N-(3-(4-phenyl-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**66**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **23,** obtained as a brown solid, yield- 72%, M.P.- 190-191 °C, $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 8.20 (s, 1H, phenyl $C_2$), 8.08 - 8.05 (m, 2H, amide NH, phenyl' $C_5$), 7.89 - 7.85 (m, 3H, phenyl' $C_2$, $C_3$, $C_6$ ), 7.55 - 7.52 (t, *J* = 7.5Hz, 1H, benzamide $C_4$), 7.48 - 7.41 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.26 (d, *J* = 6Hz, 2H, benzamide $C_3$, $C_5$), 7.22 - 7.19 (m, 1H, phenyl' $C_4$), 7.13 (d, *J* = 7Hz, 2H, benzamide $C_2$, $C_6$), 6.60 (s, 1H, pyrazole NH), 4.21(s, 1H, pyrazole $C_4$), 1.74 - 1.26 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.78 (C=O, carbonyl), 165.89 (C=O, amide), 152.70 (pyrazole $C_3$), 138.18 (phenyl $C_3$), 137.81 (phenyl $C_1$), 136.05 (phenyl' $C_1$), 134.79 (benzamide $C_1$), 131.95 (benzamide $C_4$), 128.88 (phenyl' $C_4$), 128.80 (phenyl' $C_3$, $C_5$), 128.60 (phenyl $C_5$), 128.47 (phenyl' $C_2$, $C_6$), 127.17 (benzamide $C_3$, $C_5$), 127.11 (benzamide $C_2$, $C_6$), 126.03 (phenyl $C_6$), 124.04 (phenyl $C_4$), 121.44 (phenyl $C_2$), 69.55 (pyrazole $C_5$), 57.65 (pyrazole $C_4$), 37.37, 31.63, 25.14, 23.33, 22.38 (cyclohexane

ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{27}N_3O_2$: 437.54, found –438.2181 (M+1), 439.2226 (M+2).

*N-(3-(4-(4-chlorophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**67**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **24,** obtained as a yellow solid, yield- 80%, M.P.- 210-211 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.27 (s, 1H, 4-chlorophenyl $C_5$), 8.13 (s, 1H, amide NH), 8.06 (d, *J* = 7.5Hz, 1H, 4-chlorophenyl $C_6$), 7.90 - 7.87 (m, 3H, phenyl $C_2$, 4-chlorophenyl $C_3$, $C_2$), 7.57 - 7.55 (m, 1H, benzamide $C_4$), 7.49 - 7.43 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.26 (d, *J* =7.5Hz, 2H, benzamide $C_2$, $C_6$), 7.08 (bs, 2H, benzamide $C_3$, $C_5$), 6.65 (s, 1H, pyrazole NH), 4.19 (s, 1H, pyrazole $C_4$), 1.67 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.62 (C=O, carbonyl), 165.92 (C=O, amide), 152.24 (pyrazole $C_3$), 138.02 (phenyl $C_3$), 137.86 (phenyl $C_1$), 134.74 (benzamide $C_1$), 134.72 (phenyl $C_5$), 132.94 (chlorophenyl $C_1$), 131.99 (benzamide $C_4$), 129.92 (chlorophenyl $C_4$), 128.91 (chlorophenyl $C_2$, $C_6$), 128.81 (chlorophenyl $C_3$, $C_5$), 128.69 (benzamide $C_3$, $C_5$), 127.11(benzamide $C_2$, $C_6$), 125.98 (phenyl $C_6$), 124.11 (phenyl $C_4$), 121.47 (phenyl $C_2$), 69.54 (pyrazole $C_5$), 56.93 (pyrazole $C_4$), 37.22, 31.56, 25.08, 23.33, 22.33 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{26}ClN_3O_2$: 471.99, found – 472.1797 (M+1), 474.1788 (M+3).

*N-(3-(4-(2-chlorophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**68**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **25,** obtained as a pale yellow solid, yield- 205-206 °C, M.P.- 75%, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.27 (s, 1H, 2-chlorophenyl $C_5$), 8.08 (s, 1H, amide NH), 8.05 (d, *J* = 7.5Hz, 1H, 2-chlorophenyl $C_6$), 7.91 (d, *J* = 7.5Hz, 1H, 2-chlorophenyl $C_3$), 7.87 - 7.85 (m, 2H, phenyl $C_2$, 2-chlorophenyl $C_4$), 7.54 - 7.52 (m, 1H, benzamide $C_4$), 7.48 - 7.38 (m, 4H, phenyl $C_4$, $C_5$, $C_6$, benzamide $C_3$), 7.13 (bs,

2H, benzamide $C_{2,}$ $C_6$), 6.92 (bs, 1H, benzamide $C_5$), 6.73 (s, 1H, pyrazole NH), 4.82 (s, 1H, pyrazole $C_4$), 1.75 - 1.35 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.21 (C=O, carbonyl), 165.90 (C=O, amide), 152.12 (pyrazole $C_3$), 138.05 (phenyl $C_3$), 137.83 (phenyl $C_1$), 134.77 (benzamide $C_1$), 134.20 (2-chlorophenyl $C_1$), 134.18 (phenyl $C_5$), 131.96 (benzamide $C_4$), 129.86 (2-chlorophenyl $C_2$), 129.13 (2-chlorophenyl $C_6$), 128.91 (2-chlorophenyl $C_3$), 128.81 (benzamide $C_3$, $C_5$), 128.35 (2-chlorophenyl $C_4$), 127.10 (benzamide $C_2$, $C_6$), 126.99(2-chlorophenyl $C_5$), 125.98 (phenyl $C_6$), 124.07 (phenyl $C_4$), 121.50 (phenyl $C_2$), 69.61 (pyrazole $C_5$), 53.64 (pyrazole $C_4$), 37.21, 31.40, 25.07, 23.22, 22.44 (cyclohexane ring); MS (ESI+): *m/z* calculated for $C_{28}H_{26}ClN_3O_2$: 471.99, found – 472.1692 (M+1), 474.1658 (M+3).

*N-(3-(4-(2,4-dichlorophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**69**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **26,** obtained as a pale yellow solid, yield- 82%, M.P.- 202-203 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.33 (s, 1H, 2,4-dichlorophenyl $C_2$), 8.05 (m, 2H, amide NH, 2,4-dichlorophenyl $C_5$), 7.93 - 7.88 (m, 3H, 2,4-dichlorophenyl $C_6$, phenyl $C_2$, benzamide $C_5$), 7.57 - 7.56 (m, 1H, benzamide $C_4$), 7.51 - 7.43 (m, 4H, phenyl $C_4$, $C_5$, $C_6$, benzamide $C_3$), 7.14 (d, $J$ = 8Hz,1H, benzamide $C_2$), 6.87 (d, $J$ = 8Hz, 1H, benzamide $C_6$), 6.74 (s, 1H, pyrazole NH), 4.78 (s, 1H, pyrazole $C_4$), 1.76 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.04 (C=O, carbonyl), 165.88 (C=O, amide), 151.79 (pyrazole $C_3$), 137.89 (phenyl $C_3$), 137.84 (phenyl $C_1$), 134.76 (benzamide $C_1$), 133.32 (2,4-dichlorophenyl $C_1$), 132.98 (2,4-dichlorophenyl $C_2$), 132.01 (phenyl $C_5$, benzamide $C_4$), 130.00 (2,4-dichlorophenyl $C_4$), 129.66 (2,4-dichlorophenyl $C_6$), 128.96 (2,4-dichlorophenyl $C_3$), 128.84 (benzamide $C_3$, $C_5$), 127.37 (2,4-dichlorophenyl $C_5$), 127.08 (benzamide $C_2$, $C_6$), 125.96 (phenyl $C_6$), 124.11 (phenyl $C_4$), 121.47 (phenyl $C_2$), 69.60

(pyrazole $C_5$), 53.13 (pyrazole $C_4$), 37.14, 31.40, 25.03, 23.22, 22.42 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{25}Cl_2N_3O_2$: 506.43, found – 506.1408 (M+), 508.1379 (M+2), 509.1419 (M+3).

*N-(3-(4-(4-bromophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**70**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **27,** obtained as a pale brown solid, yield- 77%, M.P.- 265-266 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.26 (s, 1H, 4-bromophenyl $C_5$), 8.08 (m, 2H, amide NH, 4-bromophenyl $C_3$), 7.91 - 7.88 (m, 3H, phenyl $C_2$, 4-bromophenyl $C_6$, $C_2$), 7.58 - 7.55 (m, 1H, benzamide $C_4$), 7.50 - 7.40 (m, 5H, phenyl $C_4$, $C_5$, $C_6$, benzamide $C_2$, $C_6$), 7.03 (d, *J* = 7Hz, 2H, benzamide $C_3$, $C_5$), 6.62 (s, 1H, pyrazole NH), 4.19 (s, 1H, pyrazole $C_4$), 1.68 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.56 (C=O, carbonyl), 165.87 (C=O, amide), 152.23 (pyrazole $C_3$), 138.01 (phenyl $C_3$), 137.85 (phenyl $C_1$), 135.25 (benzamide $C_1$), 134.77 (4-bromophenyl $C_1$), 131.99 (phenyl $C_5$), 131.64 (4-bromophenyl $C_3$, $C_5$), 130.29 (benzamide $C_4$), 128.92 (4-bromophenyl $C_2$, $C_6$), 128.82 (benzamide $C_3$, $C_5$), 127.09 (benzamide $C_2$, $C_6$), 125.99 (phenyl $C_6$), 124.09 (phenyl $C_4$), 121.43 (phenyl $C_2$), 121.10 (4-bromophenyl $C_4$), 69.49 (pyrazole $C_5$), 57.03 (pyrazole $C_4$), 37.24, 31.58, 25.09, 23.35, 22.34 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{26}BrN_3O_2$: 516.44, found – 516.8124 (M+), 518.5471 (M+2).

*N-(3-(4-(3-bromophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**71**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **28,** obtained as a pale brown solid, yield- 78%, M.P.- 262-263 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.22 (s, 1H, 3-bromophenyl$C_2$), 8.08 - 8.04 (m, 2H, amide NH, 3-bromophenyl $C_5$), 7.90 - 7.86 (m, 3H, phenyl $C_2$, 3-bromophenyl $C_6$, $C_4$), 7.54 - 7.53 (m, 1H, benzamide $C_4$), 7.49 - 7.43 (m, 3H, phenyl $C_4$,

$C_5$, $C_6$), 7.35 (d, *J* = 7.5Hz, 1H, benzamide $C_6$), 7.27 (d, *J* = 6.5Hz, 1H, benzamide $C_3$), 7.15 - 7.12 (t, *J* = 7Hz, 1H, benzamide $C_5$), 7.05 (bs, 1H, benzamide $C_2$), 6.63 (s, 1H, pyrazole NH), 4.15 (s, 1H, pyrazole $C_4$), 1.68 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.47 (C=O, carbonyl), 165.87 (C=O, amide), 152.03 (pyrazole $C_3$), 138.55 (phenyl $C_3$), 137.97 (3-bromophenyl $C_1$), 137.86 (phenyl $C_1$), 134.77 (benzamide $C_1$), 131.98 (phenyl $C_5$), 130.36 (3-bromophenyl $C_2$), 130.04 (benzamide $C_4$), 128.94 (3-bromophenyl $C_6$), 128.82 (3-bromophenyl $C_4$, benzamide $C_3$, $C_5$), 127.10 (3-bromophenyl $C_5$, benzamide $C_2$, $C_6$), 126.03 (phenyl $C_6$), 124.13 (phenyl $C_4$), 122.64 (3-bromophenyl $C_3$), 121.44 (phenyl $C_2$), 69.65 (pyrazole $C_5$), 57.28 (pyrazole $C_4$), 37.25, 31.57, 25.06, 23.33, 22.34 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{26}BrN_3O_2$: 516.44, found – 516.1281 (M+), 517.1330 (M+2), 518.1270 (M+3) and 519.1301 (M+4).

*N-(3-(4-(4-fluorophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**72**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **29,** obtained as a off white solid, yield- 78%, M.P.- 244-245 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.23 (s, 1H, 4-fluorophenyl $C_5$), 8.04 (bs, 2H, amide NH, 4-fluorophenyl $C_3$), 7.89 - 7.86 (m, 3H, phenyl $C_2$, 4-fluorophenyl $C_2$, $C_6$), 7.54 - 7.53 (m, 1H, benzamide $C_4$), 7.48 - 7.42 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.08 (bs, 2H, benzamide $C_3$, $C_5$), 6.97 - 6.94 (m , 2H, benzamide $C_2$, $C_6$), 6.58 (s, 1H, pyrazole NH), 4.20 (s, 1H, pyrazole $C_4$), 1.66 - 1.26 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.65 (C=O, carbonyl), 165.86 (C=O, amide), 162.95, 161.00 (4-fluorophenyl $C_4$), 152.59 (pyrazole $C_3$), 138.09 (phenyl $C_3$), 137.84 (phenyl $C_1$), 134.78 (benzamide $C_1$), 131.98 (phenyl $C_5$), 131.89, 131.87 (4-fluorophenyl $C_1$), 130.10, 130.07 (4-fluorophenyl $C_2$, $C_6$), 128.92 (benzamide $C_4$), 128.82 (benzamide $C_3$, $C_5$), 127.09 (benzamide $C_2$, $C_6$), 126.00 (phenyl $C_6$), 124.05 (phenyl $C_4$), 121.42 (phenyl

$C_2$), 115.48 (4-fluorophenyl $C_5$), 115.31 (4-fluorophenyl $C_3$), 69.43 (pyrazole $C_5$), 56.79 (pyrazole $C_4$), 37.22, 31.57, 25.11, 23.34, 22.36 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{26}FN_3O_2$: 455.53, found – 456.2154 (M+1), 457.2163 (M+2).

*N-(3-(4-(3-fluorophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**73**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **30,** obtained as a pale yellow solid, yield- 89%, M.P.- 260-261 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.22 (s, 1H, 3-fluorophenyl $C_2$), 8.08 (m, 2H, amide NH, 3-fluorophenyl $C_5$), 7.90 - 7.86 (m, 3H, phenyl $C_2$, 3-fluorophenyl $C_6$, $C_4$,), 7.54 - 7.53 (m, 1H, benzamide $C_4$), 7.48 - 7.42 (m, 3H, phenyl $C_4$, $C_5$, $C_6$),7.23 (d, *J* = 7Hz, 1H, benzamide $C_3$), 6.92 (d, *J* = 8Hz, 2H, benzamide $C_2$, $C_6$), 6.84 (d, *J* = 8.5Hz, 1H, benzamide $C_5$), 6.62 (s, 1H, pyrazole NH), 4.19 (s, 1H, pyrazole $C_4$), 1.67 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.54 (C=O, carbonyl), 165.86 (C=O, amide), 163.91, 161.95 (3-fluorophenyl $C_3$), 152.14 (pyrazole $C_3$), 138.72, 138.67 (3-fluorophenyl $C_1$), 138.01 (phenyl $C_3$), 137.85 (phenyl $C_1$), 134.78 (benzamide $C_1$), 131.97 (phenyl $C_5$), 129.95, 129.88 (3-fluorophenyl $C_5$), 128.93 (benzamide $C_4$), 128.81 (benzamide $C_3$, $C_5$), 127.09 (benzamide $C_2$, $C_6$), 126.01 (phenyl $C_6$), 124.33 (3-fluorophenyl $C_6$), 124.11 (phenyl $C_4$), 121.42 (phenyl $C_2$), 114.26 (3-fluorophenyl $C_2$), 114.09 (3-fluorophenyl $C_4$), 69.66 (pyrazole $C_5$), 57.34 (pyrazole $C_4$), 37.28, 31.49, 25.08, 23.34, 22.35 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{28}H_{26}FN_3O_2$: 455.53, found – 456.2085 (M+1), 457.2122 (M+2).

*N-(3-(4-(4-methoxyphenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**74**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **31,** obtained as a yellow solid, yield- 66%, M.P.- 258-259 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.27 (s, 1H, 4-methoxyphenyl $C_5$), 8.23 (s, 1H, amide NH), 8.10 (d, *J* = 7.5Hz, 1H, 4-methoxyphenyl $C_6$), 7.88 - 7.87 (m,

3H, phenyl $C_{2,}$ 4-methoxyphenyl $C_2$, $C_3$), 7.55 - 7.52(m, 1H, benzamide $C_4$), 7.46 - 7.41 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.05 (d, *J* =7Hz, 2H, benzamide $C_2$, $C_6$), 6.80 (d, *J* = 8Hz, 2H, benzamide $C_3$, $C_5$), 4.18 (s, 1H, pyrazole $C_4$), 3.75 (s, 3H, $OCH_3$), 1.66 - 1.28 (m, 10H, cyclohexane). $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.95 (C=O, carbonyl), 165.98 (C=O, amide), 158.63 (4-methoxyphenyl $C_4$), 152.95 (pyrazole $C_3$), 138.17 (phenyl $C_3$), 137.91 (phenyl $C_1$), 134.74 (benzamide $C_1$), 131.90 (benzamide $C_4$), 129.60 (phenyl $C_5$), 128.84 (4-methoxyphenyl $C_1$), 128.74 (4-methoxyphenyl $C_{2,}$ $C_6$), 128.06 (benzamide $C_2$), 127.87 (benzamide $C_6$), 127.17 (benzamide $C_3$, $C_5$), 125.94 (phenyl $C_6$), 124.10 (phenyl $C_4$), 121.56 (phenyl $C_2$), 113.88 (4-methoxyphenyl $C_{3,}$ $C_5$), 69.40 (pyrazole $C_5$), 56.82 (pyrazole $C_4$), 55.15 (-$OCH_3$), 37.20, 31.56, 25.15, 23.34, 22.36 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{29}N_3O_3$: 467.57, found – 468.2502 (M+1), 469.2343 (M+2).

*N-(3-(4-(3-methoxyphenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**75**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **32,** obtained as a pale yellow solid, yield- 75%, M.P.- 262-263 °C, $^1H$ NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.18 (s, 1H, 3-methoxyphenyl $C_5$), 8.09 (d, *J* = 8Hz, 1H, 3-methoxyphenyl $C_6$), 8.01 (s, 1H, amide NH), 7.90 - 7.86 (m, 3H, 3-methoxyphenyl $C_{2,}$ $C_4$, phenyl $C_2$), 7.56 - 7.53 (m, 1H, benzamide $C_4$), 7.49 - 7.42 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.20 - 7.17 (t, *J* = 8Hz, 1H, benzamide $C_6$), 6.76 (d, *J* = 8.5Hz, 2H, benzamide $C_3$, $C_5$), 6.68 (s, 1H, benzamide $C_2$), 6.56 (s, 1H, pyrazole NH), 4.18 (s, 1H, , pyrazole $C_4$), 3.76 (s, 3H, $OCH_3$), 1.68 - 1.29 (m, 10H, cyclohexane). $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.72 (C=O, carbonyl), 165.80 (C=O, amide), 159.65 (3-methoxyphenyl $C_3$), 152.55 (pyrazole $C_3$), 138.19 (phenyl $C_3$), 137.81 (3-methoxyphenyl $C_1$), 137.60 (phenyl $C_1$), 134.82 (benzamide $C_1$), 131.95 (phenyl $C_5$), 129.41 (benzamide $C_4$), 128.90 (3-methoxyphenyl $C_5$), 128.82 (3-methoxyphenyl $C_{6,}$

benzamide $C_3$, $C_5$), 127.09 (benzamide $C_2$, $C_6$), 126.03 (phenyl $C_6$), 123.99 (phenyl $C_4$), 121.36 (phenyl $C_2$), 112.22 (3-methoxyphenyl $C_2$, $C_4$), 69.55 (pyrazole $C_5$), 57.72 (pyrazole $C_4$), 55.17 (-$OCH_3$), 37.42, 31.52, 25.14, 23.41, 22.41 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{29}N_3O_3$: 467.57, found – 468.2283 (M+1), 469.2323 (M+2).

*N-(3-(4-(3,4-dimethoxyphenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**76**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **33,** obtained as a yellow solid, yield- 85%, M.P.- 260-261 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.25 (s, 1H, 3,4-dimethoxyphenyl $C_3$), 8.05 (m, 2H, amide NH, 3,4-dimethoxyphenyl $C_6$), 7.89 - 7.86 (m, 3H, phenyl $C_2$, 3,4-dimethoxyphenyl $C_5$, benzamide $C_5$), 7.54 (m, 1H, benzamide $C_4$), 7.48 - 7.42 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 6.77 (d, *J* = 8Hz, 1H, benzamide $C_3$), 6.69 (m, 2H, benzamide $C_2$, $C_6$) 6.55 (s, 1H, pyrazole NH), 4.16 (s, 1H, pyrazole $C_4$), 3.82 (s, 6H, -$OCH_3$ *2), 1.67 - 1.26 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.92 (C=O, carbonyl), 165.83 (C=O, amide),152.84 (pyrazole $C_3$), 148.89 (3,4-dimethoxyphenyl $C_3$), 148.12 (3,4-dimethoxyphenyl $C_4$), 138.27 (phenyl $C_3$), 137.86 (phenyl $C_1$), 134.80 (benzamide $C_1$), 131.96 (phenyl $C_5$), 128.88 (3,4-dimethoxyphenyl $C_1$, $C_6$), 128.81 (benzamide $C_3$, $C_5$), 128.55 (benzamide $C_4$), 127.08 (benzamide $C_2$, $C_6$), 125.98 (phenyl $C_6$), 123.94 (phenyl $C_4$), 121.37 (phenyl $C_2$), 111.11 (3,4-dimethoxyphenyl $C_2$, $C_5$), 69.43 (pyrazole $C_5$), 57.25 (pyrazole $C_4$), 55.92 (-$OCH_3$), 55.77 (-$OCH_3$), 37.28, 31.49, 25.18, 23.49, 22.42 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{30}H_{31}N_3O_4$: 497.60, found – 498.5027 (M+1), 499.3421 (M+2).

*N-(3-(4-(4-(trifluoromethyl)phenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**77**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **34,** obtained as a

white solid, yield- 75%, M.P.- 229-230 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.30 (s, 1H, 4-(trifluoromethyl)phenyl $C_5$), 8.11 (s, 1H, amide NH), 8.05 (d, *J* = 7.5Hz, 1H, 4-(trifluoromethyl)phenyl $C_6$), 7.91 - 7.88 (m, 3H, 4-(trifluoromethyl)phenyl $C_2$, $C_3$, phenyl $C_2$,), 7.55 - 7.54 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.50 - 7.44 (m, 3H, benzamide $C_3$, $C_4$. $C_5$), 7.27 (d, *J* = 6.5Hz, 2H, benzamide $C_2$, $C_6$), 6.73 (bs, 1H, pyrazole NH), 4.28 (s, 1H, pyrazole $C_4$), 1.72 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.52 (C=O, carbonyl), 165.95 (C=O, amide), 151.94 (pyrazole $C_3$), 140.38 (4-(trifluoromethyl)phenyl $C_1$), 137.91 (phenyl $C_3$), 137.86 (phenyl $C_1$), 134.71 (benzamide $C_1$), 132.01 (phenyl $C_5$), 129.51 (4-(trifluoromethyl)phenyl $C_2$), 129.26 (4-(trifluoromethyl)phenyl $C_6$), 128.94 (benzamide $C_4$), 128.82 (benzamide $C_3$, $C_5$), 127.10 (benzamide $C_2$, $C_6$), 125.99 (phenyl $C_6$), 125.48 (4-(trifluoromethyl)phenyl $C_3$), 125.45 (4-(trifluoromethyl)phenyl $C_5$), 125.22 (-$CF_3$), 124.17 (phenyl $C_4$), 123.06 (4-(trifluoromethyl)phenyl $C_4$), 121.51 (phenyl $C_2$), 69.77 (pyrazole $C_5$), 57.40 (pyrazole $C_4$), 37.27, 31.60, 25.04, 23.30, 22.33 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{26}F_3N_3O_2$: 505.54, found – 506.2035 (M+1), 507.2121 (M+2).

*N-(3-(4-(3-(trifluoromethyl)phenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**78**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **35,** obtained as a white solid, yield- 70%, M.P.- 230-231 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.26 (s, 1H, 3-(trifluoromethyl)phenyl $C_2$), 8.09 - 8.04 (m, 2H, amide NH, 3-(trifluoromethyl)phenyl $C_4$), 7.92 - 7.88 (m, 3H, 3-(trifluoromethyl)phenyl $C_6$, $C_5$, phenyl $C_2$), 7.58 - 7.56 (m, 1H, benzamide $C_4$), 7.51 - 7.42 (m, 6H, phenyl $C_4$, $C_5$, $C_6$, benzamide $C_3$, $C_2$, $C_6$), 7.32 (s, 1H, benzamide $C_5$), 6.68 (s, 1H, pyrazole NH), 4.28 (s, 1H, pyrazole $C_4$), 1.73 - 1.23 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.48 (C=O, carbonyl), 165.85 (C=O, amide), 152.07 (pyrazole $C_3$), 137.95 (3-(trifluoromethyl)phenyl $C_1$),

137.87 (phenyl $C_3$), 137.28 (phenyl $C_1$), 134.76 (benzamide $C_1$), 131.99 (phenyl $C_5$), 130.94 (3-(trifluoromethyl)phenyl $C_6$), 130.68 (3-(trifluoromethyl)phenyl $C_5$), 128.98 (3-(trifluoromethyl)phenyl $C_3$, benzamide $C_4$), 128.97 (3-(trifluoromethyl)phenyl $C_4$), 128.83 (benzamide $C_3$, $C_5$), 127.08 (benzamide $C_2$, $C_6$), 125.99 (phenyl $C_6$), 125.17 (3-(trifluoromethyl)phenyl $C_2$), 124.14 (phenyl $C_4$),123.01 (-$CF_3$), 121.37 (phenyl $C_2$), 69.68 (pyrazole $C_5$), 57.41 (pyrazole $C_4$), 37.22, 31.61, 25.03, 23.30, 22.33 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{26}F_3N_3O_2$: 505.54, found – 506.2057 (M+1), 507.2077 (M+2).

*N-(3-(4-(4-(trifluoromethoxy)phenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**79**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **36,** obtained as an off white solid, yield- 75%, M.P.- 245-246 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.29 (s, 1H, 4-(trifluoromethoxy)phenyl $C_5$), 8.06 (bs, 2H, amide NH, phenyl $C_2$), 7.92 - 7.88 (m, 3H, 4-(trifluoromethoxy)phenyl $C_3$, $C_2$, $C_6$), 7.58 - 7.56 (m, 1H, benzamide $C_4$), 7.51 - 7.45 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.16 - 7.12 (m, 4H, benzamide $C_2$, $C_6$, $C_3$, $C_5$), 6.65 (bs, 1H, pyrazole NH), 4.24 (s, 1H, pyrazole $C_4$), 1.70 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.54 (C=O, carbonyl), 165.87 (C=O, amide), 152.34 (pyrazole $C_3$), 148.31 (4-(trifluoromethoxy)phenyl $C_4$), 138.00 (phenyl $C_3$), 137.86 (phenyl $C_1$), 134.85(4-(trifluoromethoxy)phenyl $C_2$, $C_6$), 134.76 (benzamide $C_1$), 132.00 (phenyl $C_5$), 129.88 (-$OCF_3$), 128.94 (benzamide $C_4$), 128.83 (benzamide $C_3$, $C_5$, 4-(trifluoromethoxy)phenyl $C_1$), 127.08 (benzamide $C_2$, $C_6$), 126.01 (phenyl $C_6$), 124.10 (phenyl $C_4$), 121.44 (phenyl $C_2$), 120.88 (4-(trifluoromethoxy)phenyl $C_3$, $C_5$), 69.57 (pyrazole $C_5$), 56.91 (pyrazole $C_4$), 37.21, 31.57, 25.07, 23.33, 22.35 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{26}F_3N_3O_3$: 521.54, found – 522.4601 (M+1), 523.2424 (M+2).

*N-(3-(4-(4-cyanophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**80**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **37,** obtained as a pale yellow solid, yield- 80%, M.P.- 256-257 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.33 (s, 1H, 4-cyanophenyl $C_5$), 8.13 (s, 1H, amide NH), 8.02 (d, *J* = 7.5Hz, 1H, 4-cyanophenyl $C_6$), 7.91 - 7.88 (m, 3H, 4-cyanophenyl $C_2$, $C_3$, phenyl $C_2$), 7.58 - 7.56 (m, 3H, benzamide $C_2$, $C_6$, $C_4$), 7.50 - 7.44 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.25 (d, *J* = 6.5Hz, 2H, benzamide $C_3$, $C_5$ ), 6.73 (s, 1H, pyrazole NH), 4.24 (s, 1H, pyrazole $C_4$), 1.71 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.38 (C=O, carbonyl), 165.92 (C=O, amide), 151.55 (pyrazole $C_3$), 141.98 (4-cyanophenyl $C_1$), 137.92 (phenyl $C_3$), 137.78 (phenyl $C_1$), 134.70 (benzamide $C_1$), 132.32 (4-cyanophenyl $C_3$, $C_5$), 132.04 (phenyl $C_5$), 129.38 (benzamide $C_4$), 128.95 (4-cyanophenyl $C_2$, $C_6$), 128.83 (benzamide $C_3$, $C_5$), 127.10 (benzamide $C_2$, $C_5$), 125.96 (phenyl $C_6$), 124.21 (phenyl $C_4$), 121.51 (phenyl $C_2$), 118.80 (-CN), 111.07 (4-cyanophenyl $C_4$), 69.91 (pyrazole $C_5$), 57.63 (pyrazole $C_4$), 37.24, 31.59, 24.99, 23.29, 22.30 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{26}N_4O_2$: 462.55, found – 463.0271 (M+1), 464.1430 (M+2).

*N-(3-(4-(3-cyanophenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**81**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **38,** obtained as a pale yellow solid, yield- 75%, M.P.- 256-257 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.30 (s, 1H, 3-cyanophenyl $C_2$), 8.08 - 8.06 (m, 2H, amide NH, 3-cyanophenyl $C_6$), 7.91 - 7.89 (m, 3H, 3-cyanophenyl $C_5$, $C_4$, phenyl $C_2$,), 7.57 - 7.39 (m, 8H, benzamide $C_4$, phenyl $C_4$, $C_5$, $C_6$, benzamide $C_3$, $C_5$, $C_2$, $C_6$), 6.72 (s, 1H, pyrazole NH), 4.24 (s, 1H, pyrazole $C_4$), 1.72 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.30 (C=O, carbonyl), 165.90 (C=O, amide), 151.70 (pyrazole $C_3$), 137.98 (3-cyanophenyl $C_1$), 137.89 (phenyl $C_3$), 137.74

(phenyl $C_1$), 134.72 (benzamide $C_1$), 132.03 (phenyl $C_5$, 3-cyanophenyl $C_6$), 130.97 (3-cyanophenyl $C_2$, benzamide $C_4$), 129.35 (3-cyanophenyl $C_4$), 128.99 (3-cyanophenyl $C_5$), 128.84 (benzamide $C_3$, $C_5$), 127.10 (benzamide $C_2$, $C_6$), 125.98 (phenyl $C_6$), 124.26 (phenyl $C_4$), 121.51 (phenyl $C_2$), 118.82 (-CN), 112.64 (3-cyanophenyl $C_3$), 69.70 (pyrazole $C_5$), 57.12 (pyrazole $C_4$), 37.14, 31.62, 24.99, 23.31, 22.29 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{29}H_{26}N_4O_2$: 462.55, found – 463.3405 (M+1), 464.2121 (M+2).

*N-(3-(4-(p-tolyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**82**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **39,** obtained as a pale yellow, yield- 75%, M.P.- 255-256 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.21 (s, 1H, *p*-tolyl $C_5$), 8.12 – 8.09 (m, 2H, amide NH, *p*-tolyl $C_6$), 7.90 - 7.87 (m, 3H, *p*-tolyl $C_2$, $C_3$, phenyl $C_2$), 7.57 - 7.54 (m, 1H, benzamide $C_4$), 7.49 - 7.42 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.09 - 7.01 (m, 4H, benzamide $C_2$, $C_6$, $C_3$, $C_5$), 6.59 (s, 1H, pyrazole NH), 4.20 (s, 1H, pyrazole $C_4$), 2.29 (s, 3H, -$CH_3$), 1.67 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.83 (C=O, carbonyl), 165.88 (C=O, amide), 152.84 (pyrazole $C_3$), 138.23 (phenyl $C_3$), 137.82 (phenyl $C_1$), 136.70 (*p*-tolyl $C_4$), 134.80 (benzamide $C_1$), 132.94 (phenyl $C_5$), 131.92 (benzamide $C_4$), 129.20 (*p*-tolyl $C_3$, $C_5$), 128.85 (*p*-tolyl $C_1$), 128.78 (benzamide $C_3$, $C_5$), 128.46 (*p*-tolyl $C_2$, $C_6$), 127.12 (benzamide $C_2$, $C_6$), 126.01 (phenyl $C_6$), 124.00 (phenyl $C_4$), 121.43 (phenyl $C_2$), 69.42 (pyrazole $C_5$), 57.27 (pyrazole $C_4$), 37.33, 31.63, 25.16, 23.36, 22.39 (cyclohexane ring), 21.13 (-$CH_3$); HRMS (ESI+): *m/z* calculated for $C_{29}H_{29}N_3O_2$: 451.57, found – 452.2333 (M+1), 453.2377 (M+2).

*N-(3-(4-(o-tolyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**83**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **40,** obtained as a yellow solid, yield- 78%, M.P.- 190-191 °C,

$^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.24 (s, 1H, *o*-tolyl $C_5$), 8.08 (bs, 2H, amide NH, *o*-tolyl $C_6$), 7.91 - 7.87 (m, 3H, *o*-tolyl $C_3$, $C_4$, phenyl $C_2$), 7.57 - 7.55 (m, 1H, benzamide $C_4$), 7.50 - 7.43 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.19 (d, *J* = 6.5Hz, 1H,benzamide $C_3$), 7.12 - 7.07 (m, 2H, benzamide $C_2$, $C_6$), 6.94 (d, *J* = 7Hz, 1H, benzamide $C_5$), 6.65 (s, 1H, pyrazole NH), 4.50 (s, 1H, pyrazole $C_4$), 2.53 (s, 3H, -$CH_3$), 1.76 - 1.28 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.59 (C=O, carbonyl), 165.86 (C=O, amide), 153.81 (pyrazole $C_3$), 138.15 (*o*-tolyl $C_2$), 137.80 (phenyl $C_3$), 136.07 (*o*-tolyl $C_1$), 134.81 (phenyl $C_1$), 134.64 (benzamide $C_1$), 131.93 (phenyl $C_5$), 130.63 (benzamide $C_4$), 128.90 (*o*-tolyl $C_3$), 128.80 (benzamide $C_3$, $C_5$), 127.81 (*o*-tolyl $C_4$), 127.09 (benzamide $C_2$, $C_6$), 126.96 (*o*-tolyl $C_5$), 126.14 (o-tolyl $C_6$), 125.95 (phenyl $C_6$), 124.01 (phenyl $C_4$), 121.43 (phenyl $C_2$), 69.33 (pyrazole $C_5$), 52.94 (pyrazole $C_4$), 37.64, 31.48, 25.15, 23.37, 22.58 (cyclohexane ring), 20.28 (-$CH_3$); HRMS (ESI+): *m/z* calculated for $C_{29}H_{29}N_3O_2$: 451.57, found – 452.2315 (M+1), 453.2358 (M+2).

*N-(3-(4-(4-isopropylphenyl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**84**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **41,** obtained as a yellow solid, yield- 80%, M.P.- 240-241 °C, $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.18 (s, 1H, 4-isopropylphenyl $C_5$), 8.09 - 8.05 (m, 2H, amide NH , 4-isopropylphenyl $C_6$), 7.89 - 7.85 (m, 3H, 4-isopropylphenyl $C_2$, $C_3$, phenyl $C_2$,), 7.54 - 7.52 (m, 1H, benzamide $C_4$), 7.48 - 7.41 (m, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.11 (d, *J* = 7.5Hz, 2H, benzamide $C_3$, $C_5$), 7.03 (d, *J* = 7Hz, 2H, benzamide $C_2$, $C_6$), 6.58 (s, 1H, pyrazole NH), 4.19 (s, 1H, pyrazole $C_4$), 2.87 – 2.79 (m, 1H, -$CH_{tert}$), 1.69 - 1.26 (m, 10H, cyclohexane), 1.20 (s, 3H, -$CH_{3b}$), 1.18 (s, 3H, -$CH_{3a}$). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.83 (C=O, carbonyl), 165.85 (C=O, amide), 152.95 (pyrazole $C_3$), 147.53 (4-isopropylphenyl $C_4$), 138.24 (phenyl $C_3$), 137.81 (phenyl $C_1$), 134.80 (benzamide $C_1$), 133.12 (4-

isopropylphenyl $C_1$), 131.94 (phenyl $C_5$), 128.89(benzamide $C_4$), 128.80 (benzamide $C_3$, $C_5$), 128.40 (4-isopropylphenyl $C_2$, $C_6$), 127.11 (benzamide $C_2$, $C_6$), 126.51 (4-isopropylphenyl $C_3$, $C_5$), 126.04 (phenyl $C_6$), 124.00 (phenyl $C_4$), 121.41 (phenyl $C_2$), 69.51 (pyrazole $C_5$), 57.31 (pyrazole $C_4$), 37.33 (cyclohexane ring), 33.66 (-$CH_{tert}$), 31.63 (cyclohexane ring), 29.71, 29.67 (-$CH_{3b}$), 25.18 (cyclohexane ring), 23.95, 23.90 (-$CH_{3a}$), 23.38, 22.42 (cyclohexane ring); HRMS (ESI+): *m/z* calculated for $C_{31}H_{33}N_3O_2$: 479.62, found – 479.3470 (M+), 481.3417 (M+2).

*N-(3-(4-(naphthalen-1-yl)-1,2-diazaspiro[4.5]dec-2-ene-3-carbonyl)phenyl)benzamide* (**85**): This compound was synthesized as per general procedure described earlier in section 4.1.3.7 using compounds **65** and **42,** obtained as a pale yellow solid, yield- 70%, M.P.- 254-255 °C, $^1$H NMR (500 MHz, $CDCl_3$) $\delta_H$ 8.27 (s, 1H, naphthalen-1-yl $C_5$), 8.17 (d, *J* = 8.5Hz, 1H, naphthalen-1-yl $C_8$), 8.07 (bs, 2H, phenyl $C_2$, amide NH), 7.95 (d, *J* = 7.5Hz, 1H, naphthalen-1-yl $C_4$), 7.86 (d, *J* = 7Hz, 3H, phenyl $C_4$, $C_5$, $C_6$), 7.72 (d, *J* = 8Hz, 1H, benzamide $C_4$), 7.58 - 7.44 (m, 6H, benzamide $C_3$, $C_5$, $C_2$, $C_6$, naphthalen-1-yl $C_3$,$C_6$), 7.35 - 7.32 (t, *J* = 7.5Hz, 1H, naphthalen-1-yl $C_7$), 7.07 (d, *J* = 7Hz, 1H, naphthalen-1-yl $C_2$), 6.81 (s, 1H, pyrazole NH), 5.11 (s, 1H, pyrazole $C_4$), 1.76 - 1.25 (m, 10H, cyclohexane). $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta_C$ 186.52 (C=O, carbonyl), 165.87 (C=O, amide), 153.15 (pyrazole $C_3$), 138.14 (phenyl $C_3$), 137.86 (phenyl $C_1$), 134.78 (naphthalen-1-yl $C_1$), 134.15 (benzamide $C_1$), 132.36 (phenyl $C_5$), 132.09 (naphthalen-1-yl $C_{4a}$), 131.94 (naphthalen-1-yl $C_{8a}$), 129.03 (naphthalen-1-yl $C_5$), 128.93 (benzamide $C_4$), 128.79 (benzamide $C_3$, $C_5$), 127.76 (naphthalen-1-yl $C_3$), 127.10 (benzamide $C_2$, $C_6$), 126.41 (naphthalen-1-yl $C_4$), 126.04 (phenyl $C_6$), 125.50 (naphthalen-1-yl $C_6$), 125.36 (naphthalen-1-yl $C_7$), 125.35 (naphthalen-1-yl $C_2$), 124.08 (naphthalen-1-yl $C_8$), 123.45 (phenyl $C_4$), 121.49 (phenyl $C_2$), 69.58 (pyrazole $C_5$), 52.60 (pyrazole $C_4$), 37.34, 31.35, 25.00, 23.28, 22.68 (cyclohexane ring); HRMS

(ESI+): *m/z* calculated for $C_{32}H_{29}N_3O_2$: 487.60, found – 488.6501 (M+1), 489.1351 (M+2).

### 4.1.4 Biological evaluation

#### 4.1.4.1 *In-vitro* enzyme inhibition studies (inhibitory activity against cholinesterases)

The inhibitory potency of all the synthesized derivatives (**43-62** and **66-85**), against AChE and BuChE were determined by earlier reported protocol with minor modifications [Kumar et al. 2018b]. Briefly, stock solutions (1 mg/ml) of test compounds were prepared in DMSO. The percentage inhibitions were determined at 100 µM and 50 µM for the selection of concentration range of $IC_{50}$ assays. Six different concentrations of 0.01µM, 0.1 µM, 1 µM, 10 µM, 50 µM, and 100 µM of test compounds were used to determine $IC_{50}$. 2 µL of test or standard compounds and 100 µL of DTNB (0.0005M) were incubated in 96 well plate for 10 min. 50 µL of AChE (0.5 U $mL^{-1}$) or 50 µL of BuChE (0.5 U $mL^{-1}$) was added and incubated for 30 min. The substrate *i.e.* ATCI (for AChE, 0.00375M, 20 µL) or BTCI (for BuChE, 0.00375M, 20 µL) was added into it. Formation of yellow colored 5-thio-2-nitrobenzoate anion, as a result of the reaction of DTNB with thiocholines, was monitored for 1 min as change in absorbance at 415 nm for 20 min, on Synergy HTX multi-mode reader (BioTek, USA) against blank reading containing 2 µL DMSO instead of test compound. Donepezil (0.01–100 µM) was used as the positive control. $IC_{50}$ values were calculated using absorbance obtained from test and standard compounds. All the assays were performed in triplicate and in three independent runs.

Enzyme (AChE) kinetics study was performed to ascertain the mechanism of enzyme inhibition of compounds **44** and **67**. Seven different substrate concentrations in range of 0.25 - 5 mM were used in the study. Compounds **44** and **67** were used in three separate

concentration range of 1, 2, and 4 µM. Each concentration of test compounds was used with seven different concentrations of the substrate. The activity was measured for 10 min at an interval of 2 min in the absence and presence of test compounds. The product formed during the time frame of 10 min was calculated by Beer-Lambert law. The velocity of the enzyme reaction was obtained by plotting product formed during 10 min and $V_{max}$, $K_m$ was computed by Michaelis-Menten nonlinear regression graph and Lineweaver-Burk reciprocal linear regression plots were used to determine the mechanism of enzyme inhibition by GraphPad Prism version 5.01. $K_i$ value was determined by Dixon method in which slope of the lines from the double reciprocal Lineweaver-Burk plot was plotted as a function of test compound [Copeland and Retey 1996]. The enzyme kinetic assay was performed in triplicate.

#### 4.1.4.2 *In-vitro* blood-brain barrier permeation assay

To determine the blood-brain barrier penetration potential of compounds (**43-62** and **66-85**), parallel artificial membrane permeation assay (PAMPA-BBB) was executed[Di et al. 2003b]. Porcine brain lipid (PBL) was procured from Avanti polar lipids, alabaster and dodecane were acquired from Avra Synthesis, Hyderabad. Acceptor microplates with PVDF membrane (pore size 0.45 µm) and donor microplates were purchased from Merck Millipore. The assay was carried out by following previously reported procedure with minor modifications for BBB permeability determination [Kumar et al. 2018b]. Concisely, the acceptor plate was impregnated with 4 µL of 20 mg/ml PBL in dodecane and filled with 200 µL of buffer (pH 7.4). The acceptor plate was incubated overnight to get saturated. Compounds **43-62** and **66-85** (5 mg each), were dissolved in 1 ml of DMSO and 5 µL of the solution of compounds were taken and were further diluted with 70% DMSO and buffer of pH 7.5 to get a final concentration of 25 µg/ml. 200 µL of 25 µg/ml compounds were added to donor well plate in triplicate. The acceptor plate was

placed carefully over the donor plate like a sandwich and incubated for 18 h. After incubation, plates were carefully separated and absorbance spectra of blank (buffer, pH 7.5), donor, acceptor, and reference wells were measured with a microplate reader (HTX multi-mode reader, BioTek, USA). Each of the samples was scanned for at least five different wavelengths and in three independent runs. PAMPA model was validated with 9 commercial drugs (Verapamil HCl, Diazepam, Progesterone, Atenolol, Dopamine, Lomefloxacin, Alprazolam, Chlorpromazine and Oxazepam) whose BBB permeability has been reported earlier. *Pe* was calculated using the following equation:

$$P_e = \left(\frac{(V_D \times V_A)}{(V_D + V_A)a \times t}\right) \times In\left(1 - \frac{[Drug]_{acceptor}}{[Drug]_{equilibrium}}\right)$$

Where, $V_D$ and $V_A$ are volumes of donor and acceptor compartments respectively. *a* is the total filter area, *t* is the penetration time. $[drug]_{acceptor}$ and $[drug]_{equilibrium}$ are the absorbance of test compound at acceptor well and theoretical equilibrium absorbance respectively.

**4.1.4.3 Propidium iodide displacement assay**

To demonstrate the binding of compounds **44** and **67** to the peripheral anionic site (PAS) of AChE, propidium iodide displacement assay was performed [Peauger et al. 2017]. The assay mixture included AChE (5U) and with or without test compounds (final concentrations 10 µM and 50 µM, 150 µl), was incubated for 6 h at 25 °C. After incubation, 50 µl of propidium iodide (1 mM concentration) was added to make the total volume of 200 µl. Fluorescence intensity was measured after 10 min, at excitation and emission wavelengths of 535 nm and 595 nm, respectively using microplate reader (HTX multi-mode reader, BioTek, USA). The percentage inhibition was calculated by following equation:

$$100 - \left(\frac{IF_i}{IF_o} \times 100\right)$$

Where, $IF_i$ and $IF_o$ are the fluorescence intensities with and without inhibitor respectively. Each assay was performed as three indipendent experiments.

**4.1.4.4 Inhibition assay of $A\beta_{1-42}$ aggregation**

Metal dyshomeostasis has been suggested as a strong neurotoxic condition to induce changes in Aβ aggregation. Metal ions bind to Aβ and are involved in the production of reactive oxygen species (ROS), leading to neuronal death[Greenough et al. 2013]. Thioflavin T (ThT) assay was carried out to ascertain the inhibitory potential of compounds **44** and **67** against $Fe^{+2}$ induced $A\beta_{1-42}$ aggregation [Jan et al. 2010a]. $A\beta_{1-42}$ (Sigma) was dissolved in phosphate buffer (PBS, 10 mM, pH 7.5), compounds **44** and **67** were dissolved in DMSO. Different proportions (1:0.5, 1:1, 1:2) of the $A\beta_{1-42}$ : Inhibitor was used in the ThT assay. The final concentration of $A\beta_{1-42}$, compounds **44** and **67** and $Fe^{+2}$ was 10μM (2μL), 0.5, 10, 20μM (2μL) and 10μM (16 μL) respectively. The mixtures were incubated at room temperature for 48h under dark. After incubation period, 178 μL of 20 μM ThT was added and fluorescence intensities were measured at an excitation and emission wavelengths of 485 and 528 nm respectively.

Confocal Fluorescence Imaging: The assay mentioned in section 4.1.4.4 was further used for the confocal fluorescence imaging after 10 days of incubation. Fluorescence dye ThT; $A\beta_{1-42}$; $A\beta_{1-42}$ and ThT; $A\beta_{1-42}$ and $Fe^{+2}$; $A\beta_{1-42}$, $Fe^{+2}$ and ThT; $A\beta_{1-42}$, $Fe^{+2}$, test compound **44** or **67** and ThT; test compound **44** or **67** and ThT; test compound **44** or **67** alone were incubated and mounted on glass slide using 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma) as fixing agent. The images were taken at 40X using FITC fluorescence filter cube at excitation and emission wavelengths of

494nm and 518nm respectively. Experiments containing 10 µM of test compounds **44** and **67** were used for confocal imaging [Vyas et al. 2018].

#### 4.1.4.5 MC65 neuroprotection assay

MC65 cell lines were obtained from Dr. George M. Martin of University of Washington [Copenhaver et al. 2011, Jin et al. 2002]. The cell growth and assay was performed exactly as described previously [Kumar et al. 2018b]. Briefly, MC65 cells were grown in MEM, 5 x $10^4$ cells/well were placed in well plates and incubated with TC+ and TC- in $CO_2$ incubator. The incubated cells were further used for experiments. In the test group (50-1µM) TC was absent (TC-). The response was expressed in percentage cell viability relative to TC+ as a control. The assay was performed in triplicate and in three independent runs.

#### 4.1.4.6 Scopolamine induced amnesia model

##### 4.1.4.6.1 Materials

Scopolamine hydrobromide, donepezil (DNZ), and sodium carboxy methyl cellulose (SCMC) were purchased from Sigma-Aldrich. All other chemicals used in the present study were of analytic grade.

##### 4.1.4.6.2 Animals and housing

Adult female Swiss Albino mice, weighing 20-25 g were used in the study. The animals were housed on a 12 h light/dark cycle under controlled temperature (25 ± 2 °C) and humidity (50 ± 10%). They were allowed to acclimatize for 1 week with free access of food and water ad libitum. The food was withheld 1h before the behavioral study. All the experimental protocols were approved by the Institutional Animal Ethics Committee of the university (Banaras Hindu University, Varanasi, India) (Dean/2017/CAEC/265).

#### 4.1.4.6.3 Experimental protocol and drug administration

Animals were divided into ten groups containing 6 animals in each group i.e. (i) vehicle (1 ml) (ii) scopolamine (3mg/kg), (iii) scopolamine plus DNZ (3 mg/kg), (iv) scopolamine plus compound **44** (1.5 mg/kg), (v) scopolamine plus compound **44** (3 mg/kg), (vi) scopolamine plus compound **44** (6 mg/kg), (vii) scopolamine plus compound **67** (1.5 mg/kg), (viii) scopolamine plus compound **67** (3 mg/kg), (ix) scopolamine plus compound **67** (6 mg/kg), (x) control. The doses of compounds were fixed on the basis of their $LD_{50}$. DNZ and scopolamine hydrobromide were freshly dissolved in distilled water and test compounds in 0.5% SCMC before dosing. The route of drug administration was intraperitoneal injection (i.p) for scopolamine and oral route (p.o) for DNZ and test compounds. DNZ and compounds **44** and **67** were administered once daily in different groups for seven days. All the group animals except vehicle and control were administered with scopolamine on the seventh day to induce amnesia. The behavioral experiments were performed 5 min after scopolamine injection [Srivastava et al. 2019].

#### 4.1.4.6.4 $LD_{50}$ determination

Compounds **44** and **67** were tested for $LD_{50}$ determination according to specified protocols of OECD test guidelines for chemicals at fixed dosages of 5, 50 and 300 mg/kg. Three female Wistar rats were used for each dose. The three groups of animals containing three female rats were dosed with 5, 50, 300 mg/kg and monitored for 72 h. As per the guidelines $LD_{50}$ of compounds **44** and **67** were calculated.

#### 4.1.4.6.5 Y-Maze Test

The test was performed to evaluate the spatial working memory of all the groups. The Y-maze apparatus was of wooden made and consisted of three identical arms (labelled as A, B, and C) separated apart by an angle of 120°. Compounds **44** and **67** were

evaluated at 1.5, 3 and 6 mg/kg doses. The test was carried out on seventh day (last day) of the treatment. During the experimentation, a training session of 15 min. was performed after dosing in which animal subjects were introduced to the center of the Y-maze with closed novel arm, the animal was allowed to freely explore the arms. After 5 h of training session, main test was executed after 5 min. of scopolamine hydrobromide i.p. injection. In this session, each animal was introduced to the center of the Y-maze and allowed to move freely through the maze. The experiment was performed for 15 min. and series of arm entry of animal subject in each of the arms was recorded with a prefixed video camera. The repeated arm entry was considered as an index of memory impairment. Spontaneous alternations with three different arms in three consecutive entries (ABC, BCA, CAB not BAB) and novel arm entry were considered as the memory improvement [Kumar et al. 2018b, Mamiya and Ukai 2001, Miedel et al. 2017]. The memory improvement score was calculated using the equation:

$$\%\ Spontaneous\ \ Alternation = \frac{Number\ of\ alternations}{(total\ arm\ entries) - 2)}\ X\ 100$$

**4.1.4.7 Neurochemical analysis**

After the behavioral study, all animals were sacrificed and whole brain was isolated for neurochemical analysis. The brain was homogenized in 10 mM phosphate buffer (pH 7.4) and centrifuged for 15 min at 4350 g force at 4 °C, the supernatants were further used for the determination of AChE and catalase (CAT) levels.

AChE level was estimated in the brain of an animal by previously described methods. Briefly, 100 µL of the supernatant was incubated with 15 mM of freshly prepared ATCI (100µL) in presence of 2.7 ml of PBS for 5 min. The absorbance was recorded at 415 nm after addition of 100 µL of 1.5 mM DTNB.

CAT is an enzyme, which catalyzes the decomposition of toxic insult $H_2O_2$ produced in the body to form oxygen and water. CAT activity was determined by mixing 100 µL of supernatant with 150 µL of 0.01M PBS. The reaction was started by the addition of 250 µL of 0.16 M $H_2O_2$ followed by incubation for 1 min. at 37 ºC. 1 ml of dichromate/acetic acid solution (5% $K_2Cr_2O_7$/glacial acetic acid; 1:3 v/v) was used to stop the reaction at the end. The reaction mixture was kept on boiling water for 15 min, once the green color appeared, the absorbance was measured spectrophotometrically at 570 nm wavelength. All the experiments were performed in triplicate.

#### 4.1.4.8 *In-vivo* pharmacokinetics and brain penetration studies

Pharmacokinetics and brain penetration studies were performed using albino mice, weighing 25-30 g. All animals were housed under constant environmental conditions (22 ± 1 ℃ room temperature; 55 ± 10% relative humidity; 12 h light/dark cycle) and were allowed food and water ad libitum. Animals were fasted overnight (12 h) before dosing and continued on fasting until 4 h post administration. Thereafter, mice feed was provided ad libitum. Animals were randomly divided in two groups with five animals in each time interval. For each group, there were six-time intervals (15 min, 30 min, 1, 2, 4, and 8 h). The first group of mice received single oral dose (30 mg/kg body weight) of compound **44** (dissolved in 0.5% SCMC pre mix) and the second group animals received single oral dose (30 mg/kg body weight) of compound 67 (dissolved in 0.5% SCMC pre mix).

The blood samples (orbital-sinus puncture) and brain samples were collected earlier mentioned time intervals. Blood samples were centrifuged immediately after collection at 894 g force for 10 min at 4 °C. Plasma samples were stored at -70 °C until further analysis. Brain samples were homogenized with milli Q water. All the collected plasma

and brain samples were extracted by using HPLC grade methanol and stored for further analysis [Liu et al. 2008] [Liu et al. 2005].

#### 4.1.4.8.1 Pharmacokinetic and brain penetration analyses

Plasma and brain data were subjected to non-compartmental pharmacokinetic analysis using PK solver. The observed maximum plasma concentration (Cmax) and the time to reach the maximum plasma concentration (Tmax) were obtained by visual inspection of the experimental data. The area under the plasma concentration time curve (AUC) was calculated using linear trapezoidal method. The elimination constant (kel) was estimated by linear regression of the plasma concentrations in the log-linear terminal phase. The apparent elimination half-life (t1/2) was calculated as 0.693/kel and systemic total body clearance (Cl/F) following oral dosing was calculated as Dose/AUC0–∞. The degree of drug uptake from plasma into brain tissue was estimated from the ratio of exposure in brain over the plasma exposure (AUCbrain/AUCplasma) [Liu et al. 2005].

## 4.2 Results and Discussion

### 4.2.1 *De novo* design and *in-silico* molecular docking studies

The overall designing strategy is shown in Figure 4.2. In order to achieve successful de *novo* drug design, a fragment-growing strategy was utilized. For accomplishing this, LigBuilder 2.0 was employed (http://repharma.pku.edu.cn/ligbuilder/download.html). This package facilitates automatic build ligand molecules based on three-dimensional structure of the target protein within the binding pocket and subsequently screen them. Human acetylcholinesterase (PDB ID: 4EY7) with DNZ as co-crystallized ligand was considered as a model target. Small fragments from known ligands of single target are usually well grounded to become lead structures. The in-house small fragment library was developed on the basis of following criteria: (1) the molecular weight should be less than 250 Da, (2) lipophilic groups, and (3) The number of aromatic rings should be

equal or greater than one. In the initial stage, various in-house fragments were docked using Glide XP module of Schrödinger Maestro 2018.1. The first stage generation revealed three fragments with good binding interactions (Figure 4.3). The fragment-1 (Figure 4.3.A) showed hydrogen bonding interaction (TYR124) and hydrophobic interactions (TRP286, TYR341) at peripheral anionic site (PAS) of AChE. It also showed interaction with HIS447 residue of catalytic active site (CAS) of AChE through polar interaction. Consequently, fragment-1, which showed good docking poses was selected and used as a seed for the *de novo* designing of molecules.

As the fragments were small and their potencies were expected to be low, a second stage of seed growing was applied. Eventually, we obtained a virtual hit (compound **43**, 3,5-diarylpyrazole derivative) which had a better docking score and an improved interaction pose at the CAS and PAS of AChE (Figure 4.3.E). The obtained virtual hit was successfully synthesized and chemically modified with various electron donating (EDG, methyl and methoxy etc.) and withdrawing groups (EWG, chloro, bromo, and trifluoromethyl etc.) to establish the potencies based on biological assays. The Glide score of compound **44** was found to be -9.5 Kcal/mole. The p-chlorophenylpyrazole part of the molecule was aligned towards PAS and benzamide group of compound **44** was oriented towards CAS of AChE. The p-chlorophenyl ring interacted hydrophobically with TYR124 and formed π-π stacking interactions with TRP286 and TYR341 residues of PAS-AChE. At CAS, benzamide part formed polar interactions with HIS447 and SER203 residues. 3,5-Diarylpyrazole derivatives (**43-62**) showed satisfactory inhibitory activities and high BBB permeability. Compound **44** was found to be a suitable hit molecule (Hit compound 1) for further investigation. Based on the findings of the protein-ligand interactions of compound **44** with AChE active sites, the following modifications (and combinations thereof) were explored in the next round of

optimization step: (1) slight increase in total polar surface area (tPSA) and (2) increase in molecular weight and lipophilicity by incorporation of cyclohexane ring. The designed molecules retained the binding pose of previous hit molecules with characteristic interactions. Thus, a new series of spiropyrazoline derivatives (**66-85**) of the previous hit molecules, were designed and synthesized auspiciously.

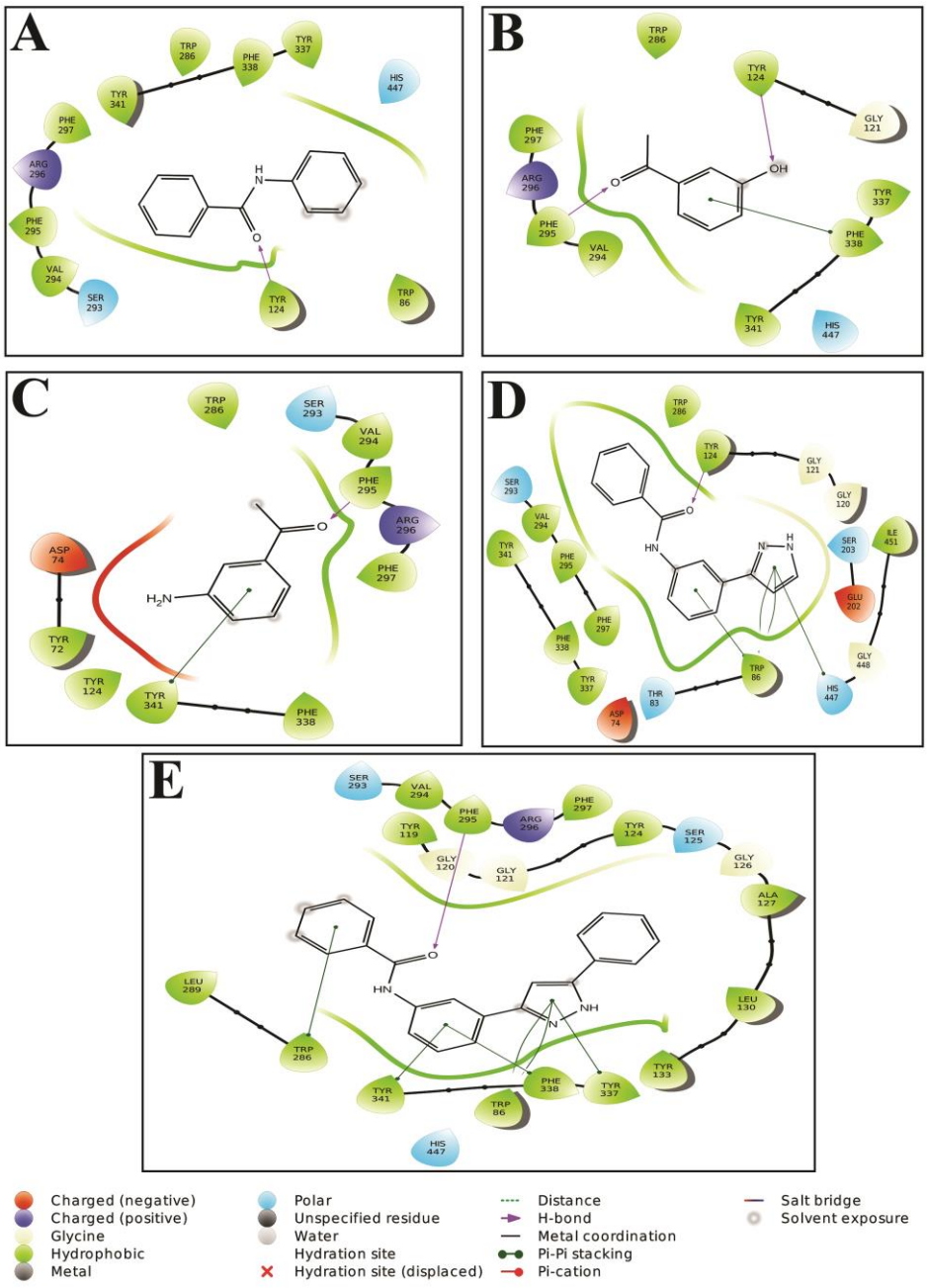


**Figure 4.3.** Docking poses of (A) fragment 1, (B) fragment 2, (C) fragment 3, (D) optimized fragment and (E) compound 43 in active site pocket of AChE (PDB: 4EY7).

The docking study of compound **67** showed better Glide score of -11.5 Kcal/mole with improved binding pose at PAS-AChE compared to compound **44**. The benzamide functionality of compound **67** was oriented towards PAS and forming hydrophobic (TYR72, TYR124,TRP286), electrostatic (ASP72), and π-π stacking interactions (TYR341) with active site residues. Moreover, spiropyrazoline —NH of compound **67** formed hydrogen bonding interaction to HIS447 and polar interaction with SER203 at CAS-AChE. Additionally, compounds **44** and **67** interacted with anionic subsite (TRP86, GLU202, PHE338), oxyanion hole (GLY120, GLY121), and acyl binding pocket (PHE295, PHE297) residues. The effective binding modes of compounds **44** and **67** are depicted in Figure 4.4. Hit compound (compound **44**) and potent optimized hit (compound **67**) were further subjected to biological evaluations.

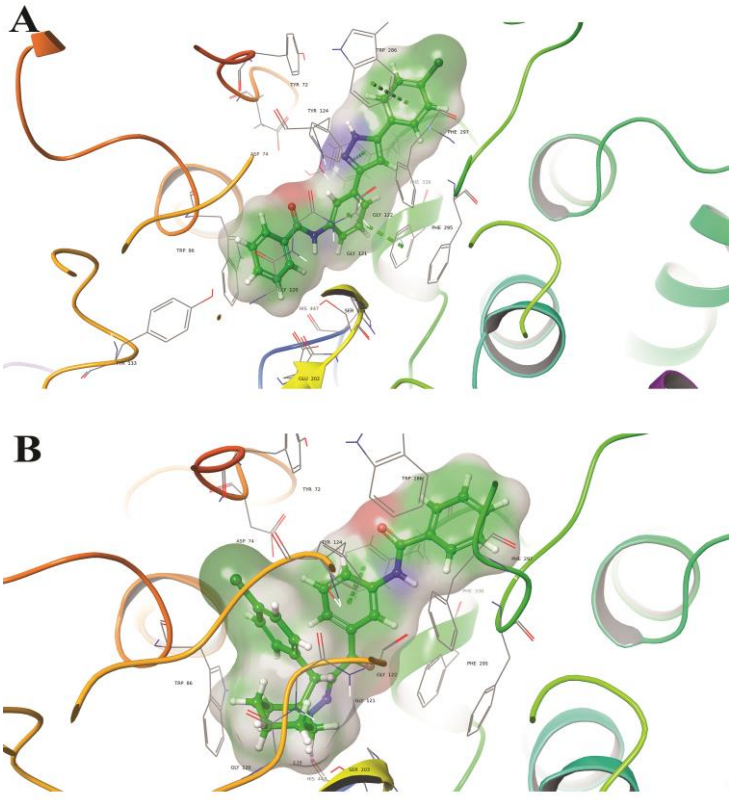


**Figure 4.4.** Binding pattern of (A) compound **44** and (B) compound **67** in active site pocket of AChE (PDB: 4EY7).

### 4.2.2 Synthetic methodology and characterization

Synthesis of 3,5-diaryl-1H-pyrazole derivatives **43-62** is illustrated in scheme 1. Formation of N-(3-acetylphenyl)benzamide (**2**) from 3-aminoacetophenone (**1**), followed by Claisen-Schmidt condensation with various substituted aromatic aldehydes, afforded corresponding key α,β-unsaturated chalcone intermediates (**23-42**). Introduction of pyrazole ring (**43-62**) system was made feasible by refluxing **23-42** with 5.0 equivalent of hydrazine hydrate in methanol, which was followed by dehydrogenation by refluxing with catalytic molecular iodine in DMSO [Kumar et al. 2014]. $^{1}H$ and $^{13}C$ NMR showed appearance of characteristic signals of $4^{th}$ position of the pyrazole ring (**43-62**) at δ 7.0 -7.2 and 95 – 105 ppm respectively.

The synthetic route for spiropyrazoline derivatives **66-85** is delineated in scheme 2. Compound **64** was prepared by reacting 1.5 equivalent of hydrazine hydrate with 4-methylbenzenesulfonyl chloride (**63**), which was refluxed with equivalent cyclohexanone to obtain cyclic ketone N-tosylhydrazone (**65)** in excellent yields. Stereoselective synthesis of spiropyrazolines was reported earlier [Liu et al. 2017, Verma et al. 2011, Wu et al. 2015]. Compound **65** underwent 1,3-cycloaddition with **23-42,** followed by a 1,3-hydrogen shift to afford spiropyrazolines (**66-85**) with high selectivity and excellent yields. In $^{1}H$ and $^{13}C$ NMR, appearance of the tertiary carbon and its corresponding proton signals at 4.0 - 4.5 and 50 – 57 ppm with other characteristic signal of derivatives established the formation of target compounds (**66-85**). Amidic –NH and pyrazole –NH were established by $D_2O$ exchange analysis. Cyclohexanone ring protons appeared as complex multiplet in aliphatic region in $^{1}H$ NMR and five distinct signals in aliphatic region of $^{13}C$ NMR spectra. Quaternary

carbon atom at C-5 position of pyrazole ring was identified at 69.0 – 69.99 ppm for all derivatives. The above said signals were further reconfirmed with 2D NMR and DEPT 135° experiments. To observe the configuration of the compound, specific rotation, differential scanning calorimetry and X-ray crystal structure analysis was performed for one representative compound **73** (3-Fluoro substituted). Specific rotation ($[\alpha]_{32.0}^{589}$) and melting temperature were observed as +213.25° (Table 4.1) and 260.4 °C (Figure 4.5) respectively. We were fortunate that compound **73** formed large crystals upon slow evaporative crystallization from ethyl acetate and ethanol (1:1), allowing for structure determination through X-ray crystallography (Figure 4.6).

**Table 4.1.** Polarimetric data of compound **73**.

| | Observed rotation $\alpha_{32.0}^{589}$ | Specific rotation[a] $[\alpha]_{32.0}^{589}$ |
|---|---|---|
| Compound **73** | +0.853 | +213.25° |

[a]Specific rotation of compound, 0.1 g in 25 ml $CH_3OH$.

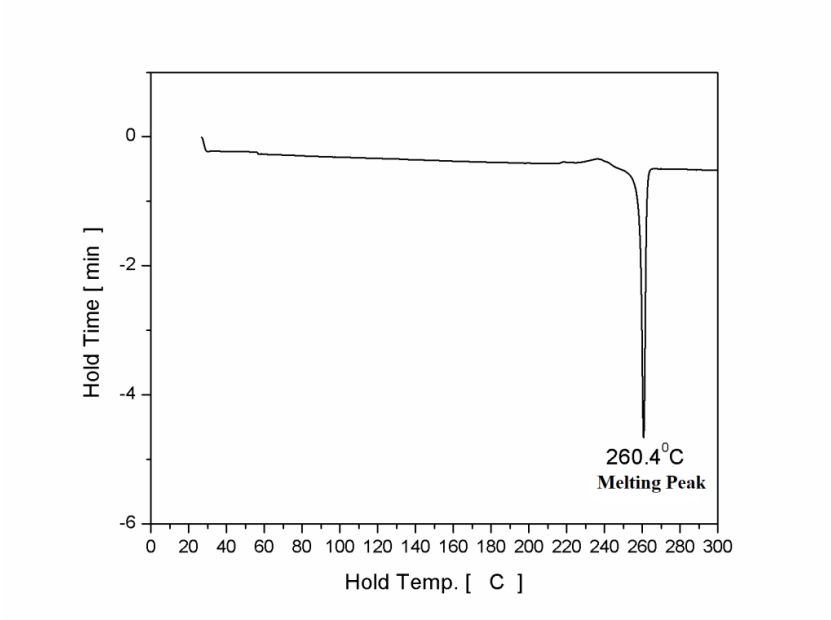



**Figure 4.5.** Differential scanning calorimetry plot of compound **73**.

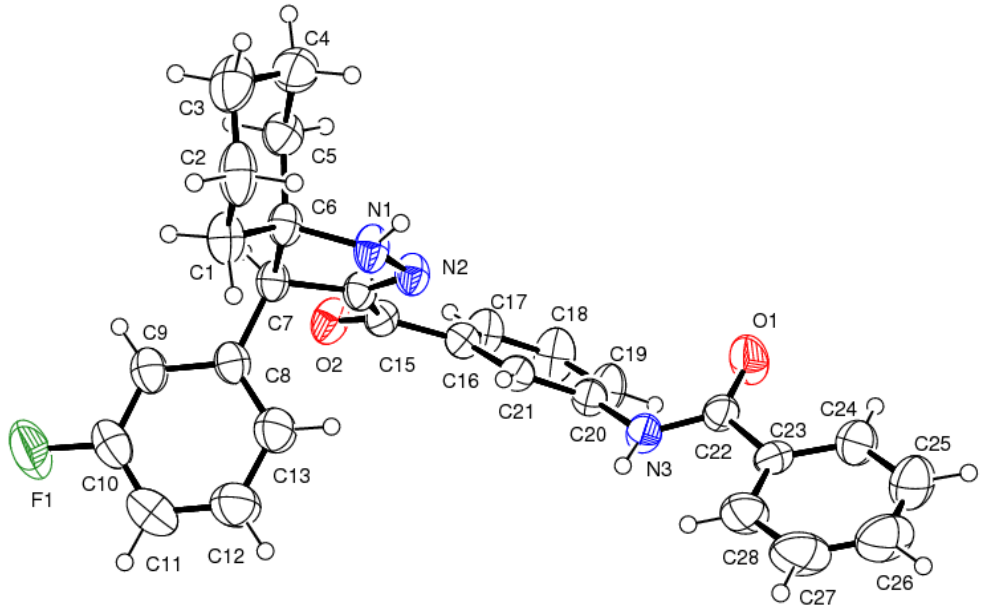



**Figure 4.6.** ORTEP diagram of compound **73** (at 40% ellipsoid level).

### 4.2.3 *In-vitro* cholinesterase inhibitory activity and SAR studies

The drug discovery and development efforts, at present, are focused on optimizing new chemical entities that act through specific enzyme inhibition. In AD, cholinergic hypothesis is an epitomized therapeutic strategy to produce effective agents [Silva et al. 2013]. The virtual hit (compound **43**) obtained from our *de novo* drug design was initially evaluated for preliminary enzyme inhibition on AChE and BuChE at concentrations of 50 and 100 µM. Our first goal was to ascertain the significance of phenyl ring at 3$^{rd}$ position of the pyrazole scaffold. Encouragingly, compound **43** showed better inhibition at these concentrations, and driving for further investigations. In the second stage, six different concentrations (0.01µM - 100 µM) of the compound were used to determine the $IC_{50}$. On AChE and BuChE, $IC_{50}$ of the compound was determined as 4.048 ± 0.115 µM and 8.633 ± 0.108 µM respectively. This may be due to the π-π interactions with PAS residue i.e TYR72. The phenyl ring attached to pyrazole was of interest for various structural modifications. Therefore, compound **43** was further explored with introduction of multiple EDG and EWG at phenyl ring. After

successful synthetic assignment, compounds **43-62** were obtained and the role of substitutions in enzyme inhibition was evaluated. Fascinatingly, out of 20 derivatives, compound **44** (*para*-chloro) was found to be potent on both AChE and BuChE with two folds increase in $IC_{50}$ (table 2; AChE = 1.937 ± 0.066 µM; BuChE = 1.166 ± 0.087

**Table 4.2.** Crystal data and structure refinement of compound **73**.

| **CCDC** | **CCDC 1866496** |
|---|---|
| Empirical formula | $C_{28}H_{26}F\ N_3O_2$ |
| Formula weight | 455.52 |
| Temperature (K) | 296(2) |
| Wavelength (Å) | 0.71073 |
| Crystal system | Orthorhombic |
| Space group | Pbca |
| a/Å | 8.6147(3) |
| b/Å | 14.7227(2) |
| c/Å | 38.3161(8) |
| α (°) | 90 |
| β (°) | 90 |
| γ (°) | 90 |
| Volume ($Å^3$) | 4859.7(2) |
| Z | 8 |
| Density $Mg/m^3$ | 1.245 |
| Absorption coefficient ($mm^{-1}$) | 0.085 |
| F(000) | 1920 |
| Crystal size $mm^3$ | 0.350 x 0.300 x 0.200 |
| Theta range for data collection (°) | 2.964 to 24.999 |
| Index ranges | -10<=h<=10, -17<=k<=17, -45<=l<=45 |
| Reflections collected | 76403 |
| Independent reflections | 4262 [R(int) = 0.0381] |
| Completeness to theta = 24.999° | 99.6% |
| Absorption correction | Semi-empirical from equivalents |
| Max. and min. transmission | 0.7453 and 0.6972 |
| Refinement method | Full-matrix least-squares on $F^2$ |
| Data / restraints / parameters | 4262 / 2 / 315 |
| Goodness-of-fit on $F^2$ | 1.059 |
| Final R indices [I>2sigma(I)] | $R_1$ = 0.0538, $wR_2$ = 0.1464 |
| R indices (all data) | $R_1$ = 0.0681, $wR_2$ = 0.1582 |
| Extinction coefficient | n/a |
| Largest diff. peak and hole ($e.Å^{-3}$) | 0.208 and -0.285 |

μM). Among the 3,5-diarylpyrazole series (**43-62**), halogen containing compounds (weak EWD groups; chloride, bromide, and fluoride) showed moderate to good $IC_{50}$ values and remaining derivatives showed satisfactory activities (Table 4.3). However, increasing the bulkiness by adding another phenyl ring as in α-napthyl (**62**, AChE = 27.78 ± 0.107 μM; BuChE = 22.30 ± 0.068 μM) exhibited highest $IC_{50}$ values. Interestingly, in all derivatives (**43-62**), *para* substituted analogs were slightly potent, when compared with other analogs. Bulky substituted analogs at *para* position (*para*-methoxy (**51**)-AChE = 8.451 ± 0.069 μM; BuChE = 7.305 ± 0.087 μM; *para*-trifluoromethoxy (**56**)-AChE = 6.092 ± 0.184 μM; BuChE = 6.755 ± 0.077 μM) of the phenyl ring showed moderate activities. Surprisingly, in spiropyrazoline series (**66-85**), same pattern of $IC_{50}$ values were observed in most of the analogs. Compound **67** (*para*-chloro) exhibited most potent inhibitory activities (AChE = 0.464 ± 0.166 μM; BuChE = 0.754 ± 0.121 μM). Compound **72** (*para*-fluoro) showed good inhibition at 0.948 ± 0.096 μM for AChE, where as in case of BuChE ($IC_{50}$ = 1.959 ± 0.082 μM), it was more than double. Compound **74** (*para*-methoxy, AChE = 2.319 ± 0.147 μM; BuChE = 3.549 ± 0.116 μM) and **79** (*para*-trifluoromethoxy, AChE = 2.240 ± 0.122 μM; BuChE = 7.792 ± 0.066 μM) derivatives of spiropyrazoline were potent in comparison to 3,5-diarylpyrazole derivatives (**51** and **56**) but not with *para*-chloro (**67**) of spiropyrazolines. Bulkiness was also not favored in spiropyrazoline analog (**85**, AChE = 29.190 ± 0.117 μM; BuChE = 35.49 ± 0.061 μM). The enzyme inhibition studies of both 3,5-diarylpyrazoles (**43-62**) and spiropyrazolines (**66-85**) on cholinesterase enzymes (AChE and BuChE) constructively developed the potency profiles of all the synthesized analogs. Weak EWD substituted derivatives showed good inhibitory activities. Compounds **44** and **67** showed significant $IC_{50}$ values among them and were also evident from molecular docking studies. The SAR studies suggested that

substitution at *para* position of phenyl ring will be beneficial for activity, whereas *ortho/meta* substitutions in the same analogs were found to be satisfactory. Further, bulkiness on the phenyl group led to decrease in activity.

To assess the selectivity towards human AChE (hAChE), four representative compounds (**44**, **46**, **67** and **69**) were selected and studied further. Compounds **44**, **46**, **67** and **69** exhibited $IC_{50}$ 1.758 ± 0.095 µM, 1.027 ± 0.062 µM, 0.472 ± 0.042 µM and 0.693 ± 0.062 µM respectively (Standard DNZ = 0.022 ± 0.031 µM). To demonstrate the worthiness of drug design approach, compounds **44** and **67** were additionally evaluated for enzyme kinetic assay on AChE.

To discover a potential analog, it is crucial that selective inhibitors be identified, which requires evaluation of candidate for binding pattern against selected target. The mechanistic role of AChE inhibition by compounds **44** and **67** was explored through enzyme kinetics parameters like maximal velocity ($V_{max}$), Michaelis–Menten / dissociation constant ($K_m$), and inhibitory concentration (ki). The reciprocal Lineweaver-Burk plot of compound **67** (Figure 4.7) suggested decreased pattern of $V_{max}$ and $K_m$ with increase in inhibitor concentrations and the intersection point of trendlines fell in the second quardrant. This result demonstrated that compound **67** inhibited the AChE enzyme non-competitively. The Dixon plot showed that it has Ki = 2.65µM (Figure 4.8).

### 4.2.4 *In-vitro* blood-brain barrier permeation assay

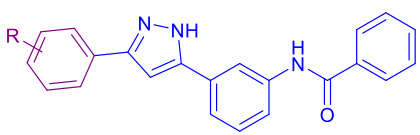
BBB and its penetration by neurotherapeutics is the gate way towards CNS activity [Di et al. 2003b]. To screen the BBB permeability of all synthesized compounds (**43-62 & 66-85**), a parallel artificial membrane permeation assay (PAMPA) was employed as described in previous reports [Kumar et al. 2018]. Nine representative commercially available drugs were selected and evaluated for BBB permeability (*Pe*, Table 4.4). The

*Pe* values of the tested compounds are listed in table 4.5. As expected, the BBB penetration potential of spiropyrazoline series (compound **67** *Pe* = 13.92 ± 0.022 x $10^{-6}$ $cms^{-1}$), was improved significantly as compared to 3,5-Diarylpyrazole series (Compound **44** *Pe* = 9.491 ± 0.34 x $10^{-6}$ $cms^{-1}$). This could be due to the introduction of lipophilic cyclohexane ring. Compound **82** (4-methyl substituted derivative) showed greater BBB permeability with *Pe* value of 14.745 ± 0.01 x $10^{-6}$ $cms^{-1}$. Moreover, all tested compounds could cross BBB *in vitro*, with excellent permeation potentials.

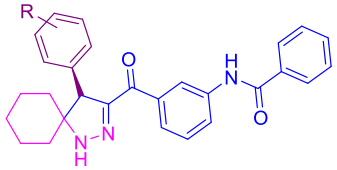
### 4.2.5 Propidium iodide displacement assay

Molecular docking studies suggested that compounds **44** and **67** demonstrated significant interactions with PAS residues. Further, enzyme inhibition and PAMPA assays manifest potent inhibition of AChE and BBB permeation. Therefore, PAS-binding affinity of compounds **44** and **67** was evaluated by propidium iodide displacement assay at 10 and 50 µM concentrations (Table 4.6). The binding of compounds **44** and **67** to PAS-AChE resulted into decreased fluorescence intensity. Compound **67** exhibited considerably equal displacement of propidium iodide at a concentration of 10 µM (21.24%) but higher in case of 50 µM (41.10%) compared to donepezil (10 µM = 21.30%; 50 µM = 38.23%). Compound **44** (10 µM = 15.68%; 50 µM = 28.04%) appeared to have lesser displacement of propidium iodide from PAS-AChE. The results of propidium iodide displacement assay are in concurrence with molecular docking studies of compounds (**44** and **67)**.

**Table 4.3.** Inhibitory potency and structures of 3,5-diaryl-1H-pyrazoles **(43-62)** and spiropyrazolines **(66-85)** derivatives.



43-62



66-85

| Comp. no | R | AChE $IC_{50}$ ± SE (µM) | BuChE $IC_{50}$ ±SE (µM) | selectivity ratio[a] | Comp. no | R | AChE $IC_{50}$ ± SE (µM) | BuChE $IC_{50}$ ±SE (µM) | selectivity ratio[a] |
|---|---|---|---|---|---|---|---|---|---|
| **43** | H | 4.048 ± 0.115 | 8.633 ± 0.108 | 0.4 | **66** | H | 1.973 ± 0.143 | 1.626 ± 0.070 | 1.2 |
| **44** | 4-Cl | 1.937 ± 0.066 | 1.166 ± 0.088 | 1.6 | **67** | 4-Cl | 0.464 ±0.166 | 0.754 ± 0.121 | 0.6 |
| **45** | 2-Cl | 3.038 ± 0.128 | 3.821 ± 0.086 | 0.7 | **68** | 2-Cl | 1.966 ± 0.104 | 2.753 ± 0.085 | 0.7 |
| **46** | 2,4-diCl | 1.945 ± 0.107 | 2.572 ± 0.121 | 0.7 | **69** | 2,4-diCl | 1.328 ± 0.107 | 2.041 ± 0.084 | 0.6 |
| **47** | 4-Br | 2.164 ± 0.095 | 1.697 ± 0.073 | 1.2 | **70** | 4-Br | 1.139 ± 0.105 | 1.533 ± 0.072 | 0.7 |
| **48** | 3-Br | 3.011 ± 0.112 | 2.345 ± 0.072 | 1.2 | **71** | 3-Br | 1.770 ± 0.110 | 2.116 ± 0.072 | 0.8 |
| **49** | 4-F | 2.090 ± 0.090 | 2.648 ± 0.060 | 0.7 | **72** | 4-F | 0.948 ± 0.096 | 1.959 ± 0.082 | 0.4 |
| **50** | 3-F | 2.579 ± 0.121 | 2.102 ± 0.078 | 1.2 | **73** | 3-F | 1.627 ± 0.097 | 1.811 ± 0.081 | 0.8 |
| **51** | 4-OMe | 8.451 ± 0.069 | 7.305 ± 0.087 | 1.1 | **74** | 4-OMe | 2.319 ± 0.147 | 3.549 ± 0.116 | 0.6 |
| **52** | 3-OMe | 8.710 ± 0.182 | 5.863 ± 0.114 | 1.4 | **75** | 3-OMe | 2.830 ± 0.172 | 3.872 ± 0.118 | 0.7 |
| **53** | 3,4-diOMe | 15.22 ± 0.128 | 9.924 ± 0.119 | 1.5 | **76** | 3,4-diOMe | 3.011 ± 0.112 | 7.279 ± 0.072 | 0.4 |
| **54** | 4-$CF_3$ | 2.360 ± 0.082 | 3.505 ± 0.115 | 0.6 | **77** | 4-$CF_3$ | 1.453 ± 0.137 | 2.385 ± 0.091 | 0.6 |
| **55** | 3-$CF_3$ | 3.196 ± 0.102 | 4.337 ± 0.059 | 0.7 | **78** | 3-$CF_3$ | 1.921 ± 0.178 | 3.670 ± 0.062 | 0.5 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **56** | 4-$OCF_3$ | 6.092 ± 0.184 | 6.755 ± 0.077 | 0.9 | **79** | 4-$OCF_3$ | 2.240 ± 0.122 | 7.792 ± 0.066 | 0.2 |
| **57** | 4-CN | 3.829 ± 0.089 | 3.811 ± 0.067 | 1.0 | **80** | 4-CN | 1.780 ± 0.090 | 2.697 ± 0.081 | 0.6 |
| **58** | 3-CN | 3.936 ± 0.091 | 4.692 ± 0.052 | 0.8 | **81** | 3-CN | 1.962 ± 0.122 | 3.694 ± 0.058 | 0.5 |
| **59** | 4-Me | 14.86 ± 0.107 | 9.625 ± 0.098 | 1.5 | **82** | 4-Me | 4.686 ± 0.096 | 8.775 ± 0.107 | 0.5 |
| **60** | 2-Me | 16.94 ± 0.067 | 18.11 ± 0.112 | 0.9 | **83** | 2-Me | 5.883 ± 0.134 | 9.460 ± 0.094 | 0.6 |
| **61** | 4-iPr | 15.49 ± 0.10 | 16.57 ± 0.077 | 0.9 | **84** | 4-iPr | 7.145 ± 0.114 | 12.74 ± 0.062 | 0.5 |
| **62** | α-Napthyl | 27.78 ± 0.107 | 22.30 ± 0.068 | 1.2 | **85** | α-Napthyl | 29.190 ± 0.117 | 35.49 ± 0.061 | 0.8 |
| **DNZ** | ----- | 0.019 ± 0.042 | 0.935 ± 0.026 | 0.2 | **--** | ---- | ---- | ---- | -- |

[a]Selectivity ratio = ($IC_{50}$ of AChE)/($IC_{50}$ of BuChE). DNZ= Donepezil

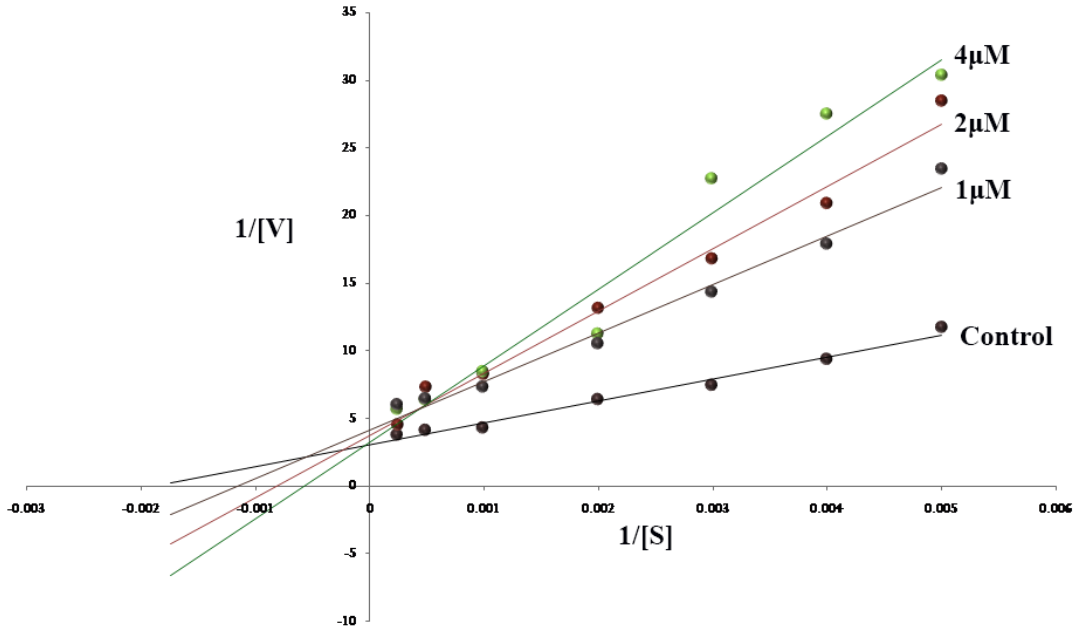



**Figure 4.7.** Lineweaver-Burk plot on three different concentrations of compound **67** for AChE: $V_{max}$, Km and $V_{max}/K_m$ at 1 µM, 2 µM and 4 µM are found to be 3.145±0.169 U/min, 0.4588±0.156 U/min, 0.2305±0.032 U/min and 0.0727±0.415 µM, 0.0086±0.003 µM, 0.0027±0.0006 µM and 43.26, 57.35, 115.25 respectively.

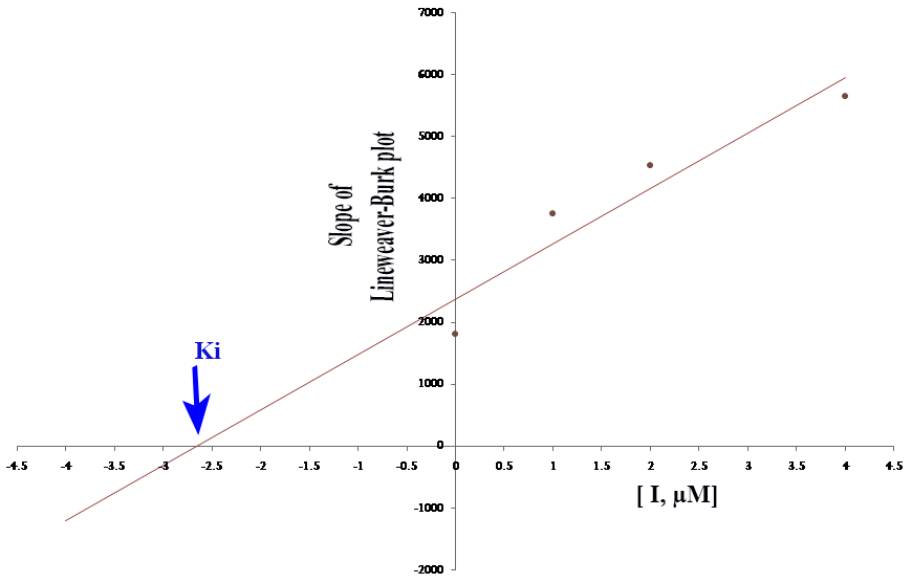



**Figure 4.8.** Dixon plot of compound **67** showing the Ki value as negative intercept on X-axis of the Dixon plot for AChE.

**Table 4.4.** Permeability (Pe×$10^{-6}$ cm/s) of 9 commercial drugs to validate the PAMPA-BBB model.

| S.no. | Compounds | Reference†(Pe×$10^{-6}$ cm/s) | Observed‡(Pe×$10^{-6}$ cm/s) |
|---|---|---|---|
| 1 | Verapamil | 16 | 20.14±0.6 |
| 2 | Diazepam | 16 | 18.75±0.7 |
| 3 | Progesterone | 9.3 | 14.35±1.2 |
| 4 | Atenolol | 0.8 | 0.69±0.2 |
| 5 | Dopamine | 0.2 | 0.18±0.1 |
| 6 | Lomefloxacin | 1.1 | 0.74±0.2 |
| 7 | Alprazolam | 5.4 | 5.6±0.4 |
| 8 | Chlorpromazine | 6.5 | 5.7±0.7 |
| 9 | Oxazepam | 10 | 9.1±1.3 |

†Reference data taken from the Di *et al.*
‡Experimental result obtained from commercial drugs, data are the mean ± SD of three independent experiments.

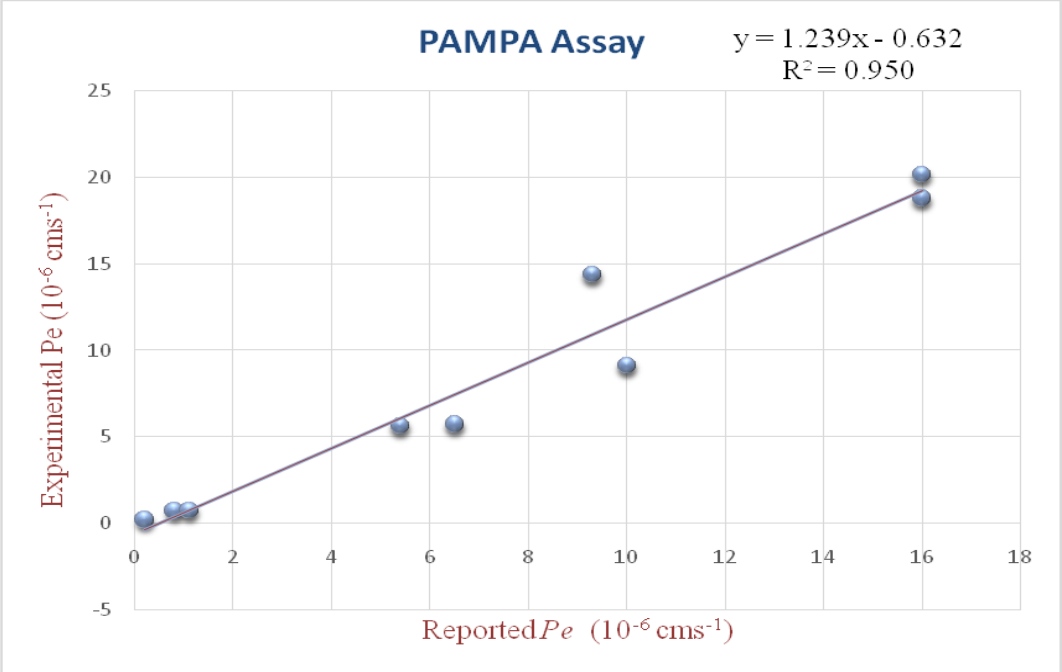



**Figure 4.9.** The linear correlation between reported and observed Pe of the commercial drugs by PAMPA assay. Pe(exp) = 1.239Pe(literature)-0.632 ($R^2$ = 0.950).
Pe (exp) ($10^{-6}$ $cms^{-1}$)> 4.324 ($10^{-6}$ $cms^{-1}$) high (CNS+) BBB permeable,
Pe (exp)($10^{-6}$ $cms^{-1}$) in between 4.324 - 1.846 ($10^{-6}$ $cms^{-1}$) BBB permeability unpredictable (CNS±). Pe (exp) < 1.846 ($10^{-6}$ $cms^{-1}$) low (CNS-) BBB permeable.

**Table 4.5.** Permeability Pe ($10^{-6}$ cm $s^{-1}$) results from the PAMPA-BBB assay of synthesized compounds and their prediction of BBB Penetration.

| Comp.no | Pe ($10^{-6}$ cm $s^{-1}$)[a,b] | Comp.no | Pe ($10^{-6}$ cm $s^{-1}$)[a,b] |
|---|---|---|---|
| **43** | 9.655±0.04 | **66** | 13.750±0.01 |
| **44** | 9.491±0.34 | **67** | 13.920±0.02 |
| **45** | 9.513±0.23 | **68** | 13.855±0.60 |
| **46** | 9.990±0.01 | **69** | 14.175±0.02 |
| **47** | 9.729±0.07 | **70** | 13.579±0.01 |
| **48** | 9.694±0.05 | **71** | 13.654±0.04 |
| **49** | 9.606±0.08 | **72** | 13.764±0.09 |
| **50** | 9.627±0.09 | **73** | 13.698±0.06 |
| **51** | 9.656±0.06 | **74** | 13.830±0.06 |
| **52** | 9.748±0.04 | **75** | 13.659±0.03 |
| **53** | 9.925±0.01 | **76** | 14.133±0.01 |
| **54** | 9.964±0.04 | **77** | 14.356±0.04 |
| **55** | 9.748±0.03 | **78** | 14.656±0.02 |
| **56** | 9.367±0.03 | **79** | 13.656±0.02 |
| **57** | 9.369±0.01 | **80** | 14.340±0.02 |
| **58** | 9.664±0.02 | **81** | 14.241±0.05 |
| **59** | 9.731±0.04 | **82** | 14.745±0.01 |
| **60** | 9.627±0.09 | **83** | 13.552±0.03 |
| **61** | 9.760±0.05 | **84** | 13.974±0.04 |
| **62** | 9.631±0.08 | **85** | 14.156±0.04 |

[a]Data are expressed as the standard deviation (SD) of three independent experiments.
[b]Compounds with $Pe > 4.324 \times 10^{-6}$ cm $s^{-1}$ could cross the BBB (CNS+). Compounds with $Pe < 1.846 \times 10^{-6}$ cm $s^{-1}$ could not cross the BBB (CNS−), and compounds with $1.846 \times 10^{-6}$ cm $s^{-1} < Pe < 4.324 \times 10^{-6}$ cm $s^{-1}$show uncertain BBB permeation (CNS±); All compounds could cross the BBB.

### 4.2.6 $A\beta_{1-42}$ aggregation assay (Thioflavin T assay) and confocal fluorescence imaging

Compounds **44** and **67** were further evaluated by metal induced $A\beta_{1-42}$ aggregation assay to establish their potency. $A\beta_{1-42,}$ when incubated with the metal showed 100% aggregation. DNZ, at a dose of 20 µM, showed significant inhibition of metal induced $A\beta_{1-42}$ aggregation. Compound **44**, inhibited nearly 50% of $A\beta_{1-42}$ aggregation, whereas in compound **67** it was more than 50% compared with $Fe^{+2}$ + $A\beta_{1-42}$ and $A\beta_{1-42}$ groups (Figure 4.10.A). Recently, Avinash S. Kumbhar and coworkers demonstrated the use of Thioflavin T (ThT) based confocal imaging experiments to monitor conformational changes of $A\beta_{1-42}$ aggregation in presence of Ru(II) polypyridyl complexes [Vyas et al. 2018] . Confocal imaging was carried out to understand the interaction of $A\beta_{1-42}$, $Fe^{+2}$, compounds **44**, and **67** at molecular level after 10 days of incubation. Fluorescent background was obtained using ThT dye (Figure 4.10.B). $A\beta_{1-42}$ aggregates were obtained when incubated and treated with ThT (Figure 4.10.C), whereas no fluorescence was observed in $A\beta_{1-42}$ alone (Figure 4.10.D) and $A\beta_{1-42}$ along with the metal were incubated without ThT (Figure 4.10.E). These blank images explain that neither $A\beta_{1-42}$ and metal nor their combinations showed any background noises in absence of ThT. $A\beta_{1-42}$ incubated with metal showed vigorous plaques deposition (Figure 4.10.F), while the plaques were decreased upon treatment with compounds **44** and **67** (Figure 4.10.G and 4.10.H). These results suggest that test compounds possibly inhibit or decrease the $A\beta_{1-42}$ aggregation at early stages of fibril formation.

**Table 4.6.** Propidium iodide displacement assay.

| Comp.no | Displacement of Propidium iodide from AChE PAS (% inhibition)[a] | |
|---|---|---|
| | At 10 μM | At 50 μM |
| **44** | 15.68 ± 1.96 | 28.04 ± 2.81 |
| **67** | 21.24 ± 2.18 | 41.10 ± 2.49 |
| **Donepezil** | 21.30 ± 1.69 | 38.23 ± 3.37 |

[a]Data are expressed as the standard deviation (SD) of three independent experiments.

### 4.2.7 Neuroprotection studies on MC65 cell lines

Cell line studies provide a system for ready and rapid evaluation [Allen et al. 2005b]. The use of cell cytotoxicity analysis is a valuable tool to study issues of clinical relevance, especially those related to diseases, and cell toxicity mechanisms. MC65 is a versatile *in vitro* model in neurobiology and it conditionally expresses a C-terminal derivative of the amyloid β precursor protein (AβPP) termed SβC (a fusion protein composed of the amino-17 and carboxyl-99 residues of βPP), which further induces ROS generation. The cell line is accompanying with oxidative stress and Aβ induced cellular toxicity in tetracycline (TC) removal condition (TC-) [Shastry et al. 2001]. Neurotherapeutic likeliness and toxicity profiles of the potent derivatives (compounds **44** and **67**) were ascertained by MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Compounds **44** and **67,** at concentrations of 50, 40, 30, 20, 10, and 1μM, were used in the experiment and compared to that of tetracycline (TC+). Significant decrease in the Aβ production was observed with respect to TC- cells (Figure 4.11).

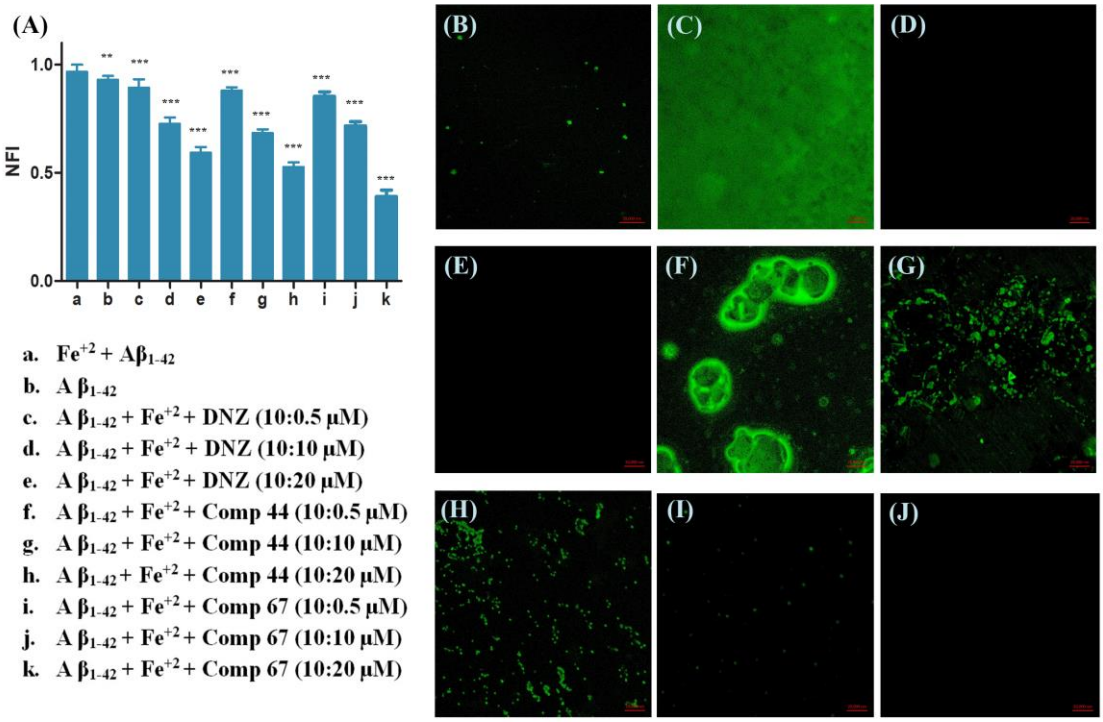



**Figure 4.10.** $A\beta_{1-42}$ aggregation Inhibition assay and confocal imaging analysis: (A) metal induced $A\beta_{1-42}$ aggregation assay (One-way ANOVA followed by Newman-Keuls multiple comparison test compare all pair of columns *** $p < 0.0001$), error bars represent the standard deviation (SD) of the normalized fluorescence intensity (NFI), donepezil (DNZ)); Confocal image of (B) Thioflavin T (ThT) (C) $A\beta_{1-42}$ along with ThT (D) $A\beta_{1-42}$ without fluorescence dye ThT (E) $A\beta_{1-42}$ along with $Fe^{+2}$ (F) $A\beta_{1-42}$ containing $Fe^{+2}$ and ThT (G) $A\beta_{1-42}$ containing $Fe^{+2}$, compound **44** and ThT (H) $A\beta_{1-42}$ containing $Fe^{+2}$, compound **67** and ThT (I) compound **67** and ThT (J) containing only compound **67.** Scale size: 20000 nm.

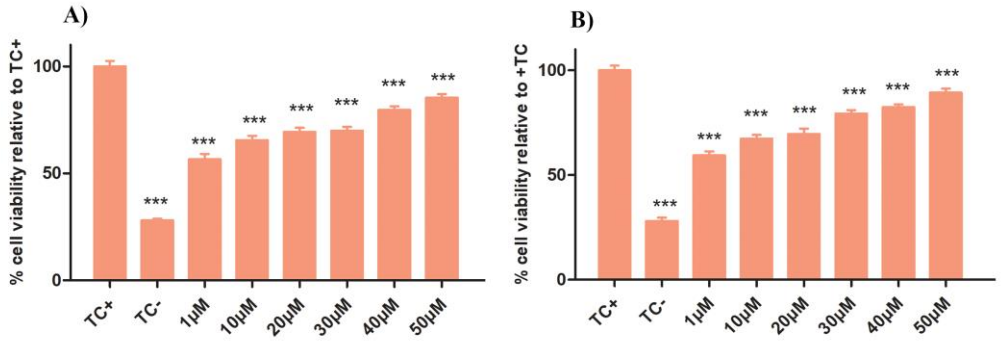



**Figure 4.11.** Neuroprotection assay on MC65 cell lines with A) Compound **44** B) Compound **67**; MC65 cells were treated with Compounds **44** and **67** at mentioned concentrations in the absence of Tetracycline (TC-). TC+ was taken as control (One-way ANOVA followed by Newman-Keuls multiple comparison test compare all pair of columns *** $p < 0.0001$).

### 4.2.8 *In-vivo* behavioral studies

The evaluation of a chemical molecule for potential *in-vivo* efficacy requires a robust approach, which screens the efficacy in appropriate cognitive domains and monitors behavior to appraise cognition. The spontaneous alternation behavior in Y-maze, as sign of short-term memory, is a fruitful method to screen new molecules against amnesic rodent model [Miedel et al. 2017]. The effect of compounds **44** and **67** on scopolamine-induced impairment of spontaneous alternation behavior and number of arm entries were assessed by administering scopolamine hydrobromide (3 mg/kg) by intraperitoneal injection. The dose of the compounds was fixed at half of the lethal dose ($LD_{50}$) (Table 4.7 to 4.12). Scopolamine hydrobromide showed markedly impaired spontaneous alternation behavior and significant difference ($p < 0.05$) with respect to control, vehicle and DNZ treated groups. (Figure 4.12.A). Compound **44,** at a dose of 1.5 mg/kg, did not exhibit no significant difference with scopolamine hydrobromide treated group, whereas at doses of 3mg/kg and 6 mg/kg showed significant difference. Notable differences were observed among compound **67** treated groups, relative to the Scopolamine hydrobromide 3mg/kg group. There was a dose dependent increase in the percentage of spontaneous alternation among compound **44** treated groups. No significant difference in the percentage of spontaneous alternation was observed between DNZ treated group and compound **67** (at dose of 3 mg/kg and 6 mg/kg) treated groups. Neophobia and recognizing behaviors of all groups were assessed by monitoring their novel arm entries. Scopolamine hydrobromide (3 mg/kg) showed remarkable decrease in novel arm entries whereas, DNZ (3 mg/kg) exhibited no significant deference with respect to control and vehicle groups. Compound **44** at a dose of 1.5 mg/kg showed significant difference with respect to scopolamine and DNZ groups. Dose dependent increase in novel arm entries was observed until a dose of 3mg/kg, before hitting a plateau at

6mg/kg. Same pattern was observed with compound **67,** which at a dose of 3 mg/kg showed no significant difference compared to DNZ. Increase in novel arm entries by spiropyrazoline derivative (compound **67** at 3mg/kg) was mainly because of greater availability of drug in the brain as compared to 3,5-diarylpyrazole analog (compound **44)**. In case of % total arm entries, all groups are significantly different with respect to scopolamine group. This suggests that none of the compounds influenced the locomotor activity of the animals.

**Table 4.7.** Protocol for $LD_{50}$ determination the compound **44**.

| | |
|---|---|
| **Test substance**<br>1. Physical nature<br>2. Code | <br>Solid<br>PTH-02 |
| Vehicle | 0.5% Sodium carboxy methyl cellulose (SCMC) |
| **Test animals**<br>1. Sex<br>2. Number | Rat<br>Female<br>3 |
| **Test conditions**<br>1. Starting Dose<br>2. Dosing volumes<br>3. Time & date of dosing | <br>300 mg/kg<br>0.5 ml<br>10 AM 05/06/2018 |

**Table 4.8.** Effect of compound **44** on the body wt. of the animals at the dose of 300 mg/kg.

| Group | Body wt. (gm) on 05/06/2018 at 10 AM | Body wt. (gm) on 06/06/2018 at 10AM | Body wt. (gm) on 07/06/2018 at 10 AM |
|---|---|---|---|
| 1 | 220 | 216 | 216 |
| 2 | 210 | 208 | 207 |
| 3 | 205 | 205 | 205 |

**Table 4.9.** The onset of toxicity with compound **44** in the period of 72 h.

| Group | Body wt. changes (gm) | | | Onset of toxicity | Reversibility | Date & time of death |
|---|---|---|---|---|---|---|
| | 05/06/2018 | 06/06/2018 | 07/06/2018 | | | |
| 1 | 00 | 04 | 00 | 05/06/2018, 4 PM | No | 07/06/2018, 6PM |
| 2 | 00 | 02 | 01 | 05/06/2018, 4 PM | No | 07/06/2018, 3PM |
| 3 | 00 | 00 | 00 | 05/06/2018, 4 PM | No | 07/06/2018, 6PM |

**Rationale for the selection of the starting dose:** No animal death was observed at 5mg/kg and 1 death at 50mg/kg.

**Discussion and interpretation of results:** Animals were dosed as per the OECD guideline 423 (Acute Oral Toxicity – Acute Toxic Class Method) at 5mg/kg, 50 mg/kg and 300 mg/kg doses. All animals are died at 300mg/kg dose within 72 hrs.

**Conclusions:** As per OECD guideline (Annex 2b) **$LD_{50}$ = 200mg/kg**.

**Table 4.10.** Protocol for $LD_{50}$ determination the compound **67**.

| | |
|---|---|
| **Test substance**<br>3. Physical nature<br>4. Code | <br>Solid<br>SPTH-02 |
| Vehicle | 0.5% Sodium carboxy methyl cellulose (SCMC) |
| **Test animals**<br>3. Sex<br>4. Number | Rat<br>Female<br>3 |
| **Test conditions**<br>4. Dose<br>5. Dosing volumes<br>6. Time & date of dosing | <br>300 mg/kg<br>0.5 ml<br>10 AM 05/06/2018 |

**Table 4.11.** Effect of compound 67 on the body wt. of the animals at the dose of 300 mg/kg.

| Group | Body wt. (gm) on 05/06/2018 at 10 AM | Body wt. (gm) on 06/06/2018 at 10AM | Body wt. (gm) on 07/06/2018 at 10 AM |
|---|---|---|---|
| 1 | 200 | 196 | 196 |
| 2 | 206 | 202 | 202 |
| 3 | 212 | 210 | 210 |

**Table 4.12.** Onset of toxicity with compound **67** in the period of 72 h.

| Group | Body wt. changes (gm) | | | Onset of toxicity | Reversibility | Date & time of death |
|---|---|---|---|---|---|---|
| | 05/06/2018 | 06/06/2018 | 07/06/2018 | | | |
| 1 | 00 | 04 | 00 | 05/06/2018, 6 PM | No | 07/06/2018, 2PM |
| 2 | 00 | 04 | 00 | 05/06/2018, 6PM | No | 07/06/2018, 6PM |
| 3 | 00 | 02 | 00 | 05/06/2018, 6 PM | No | 07/06/2018, 6PM |

**Rationale for the selection of the starting dose:** No animal death was observed at 5mg/kg and 1 death at 50mg/kg.

**Discussion and interpretation of results:** Animals were dosed as per the OECD guideline 423 (Acute Oral Toxicity – Acute Toxic Class Method) at 5mg/kg, 50 mg/kg and 300 mg/kg doses. All animals are died at 300mg/kg dose within 72 hrs.

**Conclusions:** As per OECD guideline (Annex 2b) **$LD_{50}$ = 200mg/kg**.

### 4.2.9 Neurochemical level estimation

The animals used in the behavioral study were further used to evaluate the neurochemical (AChE) level in brain by earlier described protocols [Kumar et al. 2018b]. High levels of AChE were observed in scopolamine treated animals (Figure 4.13.A), but was significantly low with DNZ treatment. Compounds **44** (3 mg/kg) showed slightly higher levels of AChE as compared to DNZ and compounds **67** (3 mg/kg). Catalase (CAT) is a very important enzyme in protecting the cells from oxidative damage by reactive oxygen species (ROS). CAT levels in the brain of animals were also determined after the behavioral studies (Figure 4.13.B) and compounds **44** and **67** maintained its normal level at a dose of 3 mg/kg (Figure 4.13). The brain tissue pattern in the normal, toxic and treated groups was further evaluated by histopathological examination (Figure 4.12.E) of the brain samples. Some abnormal cell morphology was observed in the scopolamine treated animal, while the standard pattern of the brain tissues in control, compounds **44** and **67** treated animals evidently deduce that the test compounds were safer for brain tissues.

### 4.2.10 *In-vivo* mice brain pharmacokinetic profiles of compounds 44 and 67

For a potent CNS drug, it is necessary to understand the brain penetration and ratio of brain and plasma concentration [Liu et al. 2008]. In view of the appreciative potency in *in-vitro* BBB permeation and scopolamine induced amnesia models, compounds **44** and **67** were further evaluated for brain pharmacokinetic profile in mice. As shown in Table 4.13, compound **67** showed better brain penetration and it was increased by 2.05 times

compared to compound **44**. Moreover, it showed acceptable terminal half-life ($t_{1/2}$ = 2.61 h) after oral administration.

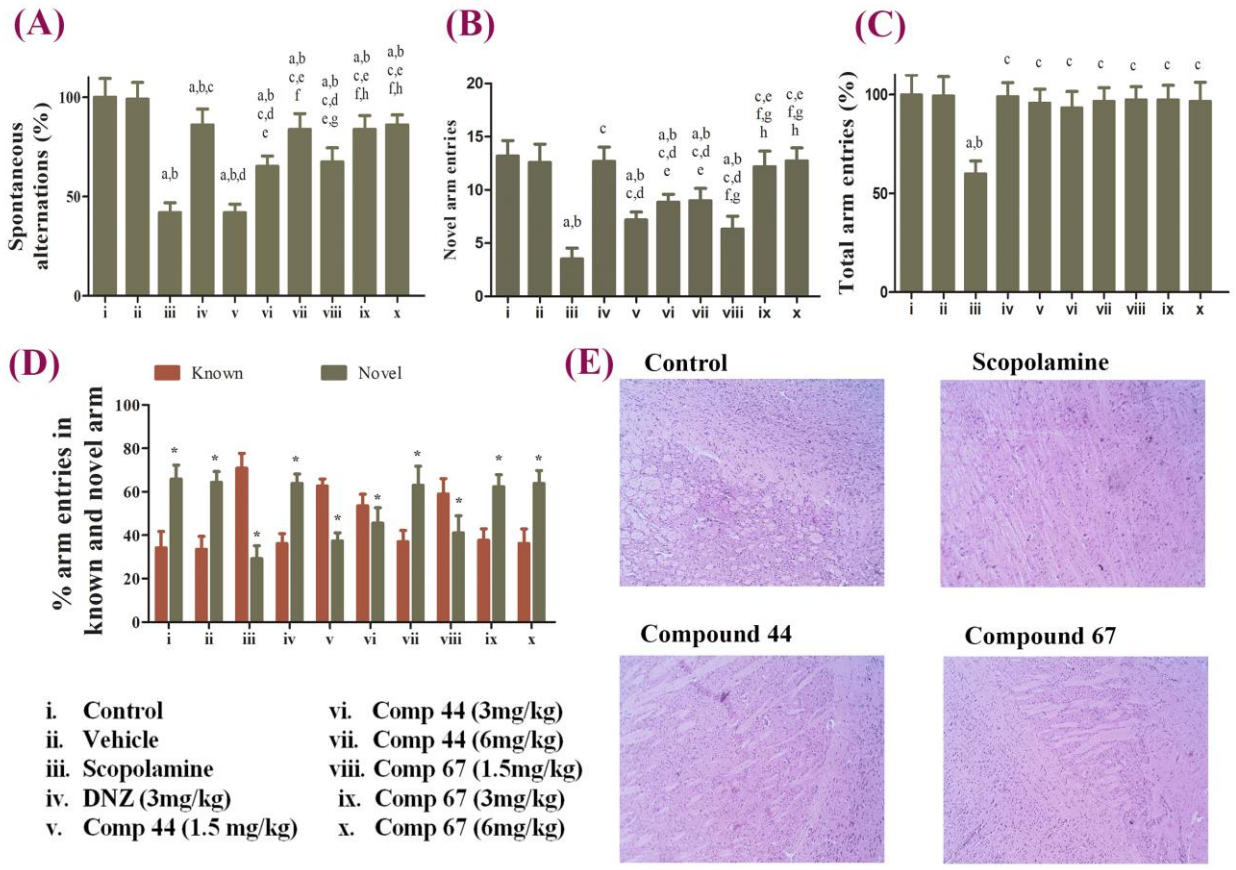



**Figure 4.12.** Effect of compounds **44** and **67** on scopolamine-induced impairment of spontaneous alternation behavior (A) spontaneous alternation score (spontaneous alternation %); (B) Novel arm entries of the animals; (C) Total arm entries % in the Y-maze test. (E) Histopathology of brain samples. Bars shows data as Mean±SD, n = 6, $^{a}p < 0.05$ compared to control; $^{b}p < 0.05$ compared to vehicle; $^{c}p < 0.05$ compared to scopolamine; $^{d}p < 0.05$ compared to donepezil (DNZ) at dose of 3 mg/kg; $^{e}p < 0.05$ compared to compound **44** at dose of 1.5 mg/kg; $^{f}p < 0.05$ compared to compound **44** at dose of 3 mg/kg; $^{g}p < 0.05$ compared to compound **44** at dose of 6 mg/kg; $^{h}p < 0.05$ compared to compound **67** at dose of 1.5 mg/kg; $^{i}p < 0.05$ compared to compound **67** at dose of 3 mg/kg (One-way ANOVA followed by Newman - Keuls test).

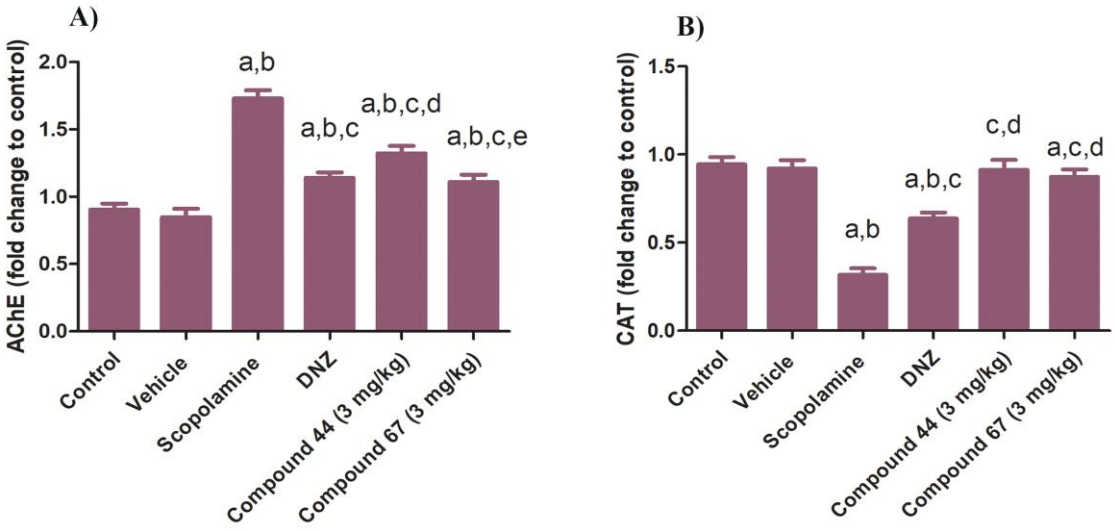
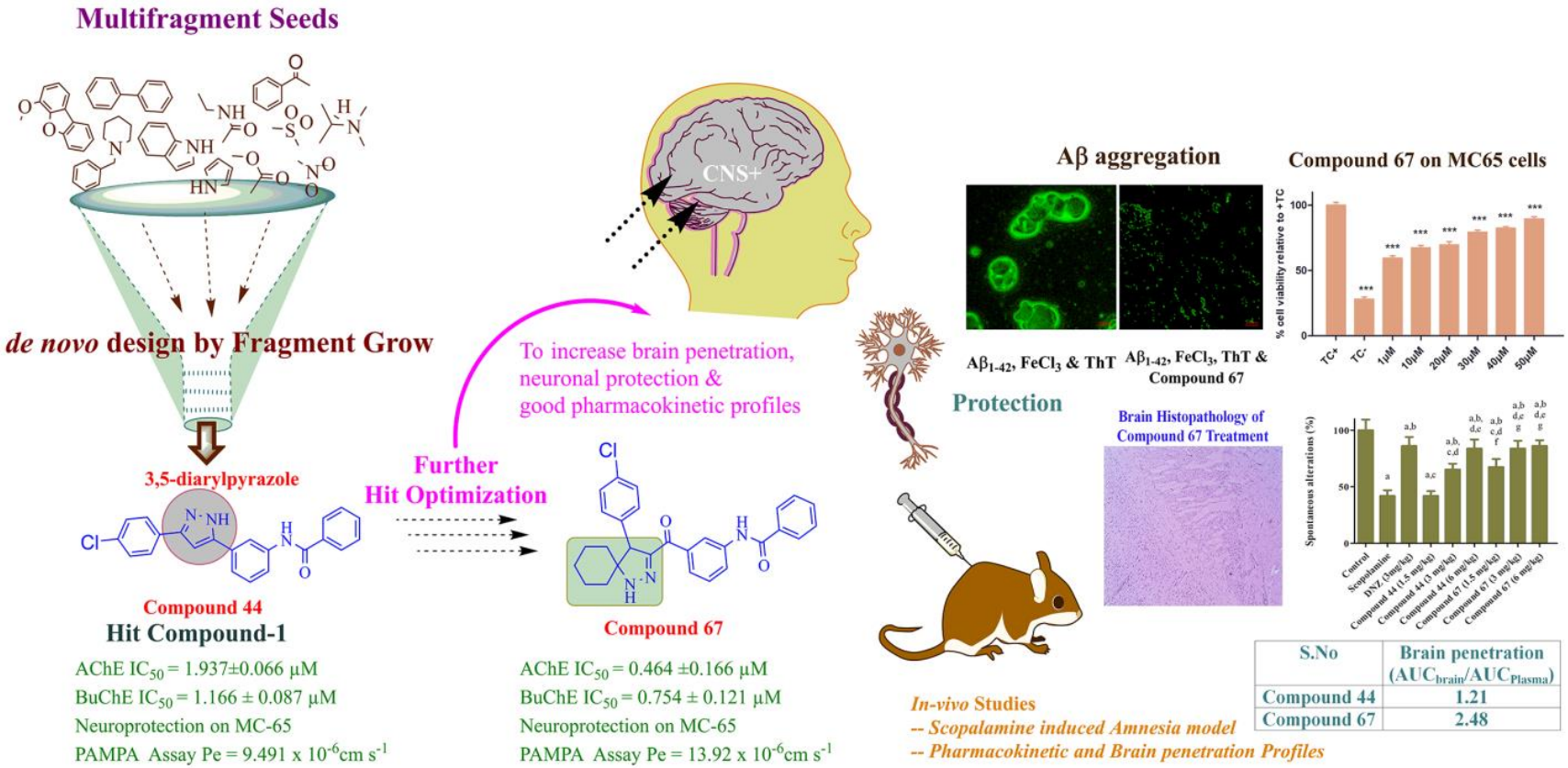



**Figure 4.13.** Analysis of AChE and Catalase levels **(A)** Estimation of AChE level. **(B)** Estimation of AChE CAT levels; (Mean±SD, n = 6, [a]p< 0.05 compared to control; [b]p< 0.05 compared to vehicle; [c]p< 0.05 compared to scopolamine; [d]p< 0.05 compared to DNZ; [e]p < 0.05 compared to compound **44** at dose of 3 mg/kg (One-way ANOVA followed by Newman - Keuls test).

**Table 4.13.** Pharmacokinetics and brain penetration parameters of compound **44** and **67** following oral administration (30 mg/kg) to mice.[a]

| Parameter | Compound 44 | | Compound 67 | |
|---|---|---|---|---|
| | Plasma | Brain | Plasma | Brain |
| $C_{max}$ (µg/mL) | 10.21 ± 2.24 | 4.12 ± 0.57 | 28.15 ± 3.45 | 40.12 ± 5.18 |
| $T_{max}$ (h) | 0.25 | 1 | 0.25 | 1 |
| $AUC_{0-t}$ (µg min/mL) | 6.77 ± 0.75 | 8.17 ± 0.97 | 57.11 ± 6.54 | 141.60 ± 19.78 |
| $t_{1/2}$ (h) | 0.60 ± 0.04 | 1.83 ± 0.09 | 1.68 ± 0.13 | 2.61 ± 0.34 |
| MRT 0-inf_obs (h) | 0.96 ± 0.08 | 3.10 ± 0.34 | 2.33 ± 0.16 | 3.75 ± 0.32 |
| Cl/F (mL/kg/h) | 383.2 ± 28.62 | 263.8 ± 14.47 | 49.9 ± 4.87 | 18.6 ± 2.45 |
| Vz/F (mL/kg) | 333.2 ± 42.95 | 695.7 ± 57.85 | 121.05 ± 16.45 | 70.2 ± 3.72 |
| Brain penetration ($AUC_{brain}/AUC_{Plasma}$) | 1.21 | | 2.48 | |

[a]Data are presented as mean±S.D. (n = 5 per each time interval). Significant difference ($p<0.05$); $T_{max}$, peak time; $C_{max}$, peak concentration; AUC, the extrapolated area under the plasma concentration−time curve; $t_{1/2}$, terminal half-life; MRT, mean resident time; CL, total plasma clearance; $V_z$ volume of distribution.




| S.No | Brain penetration ($AUC_{brain}/AUC_{Plasma}$) |
|---|---|
| Compound 44 | 1.21 |
| Compound 67 | 2.48 |

**Figure 4.14.** Overview of development of pyrazole and spiropyrazoline analogs.