Chapter 4

Multitargeted Novel Piperazindiones for Management of AD

4.1. Experimental Work

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

The compounds for the study were designed by Hybrid pharmacophore approach and database mining. The two compounds *i.e.* Donepezil (FDA approved, centrally acting AChE inhibitor) and PQ912 (phase II Clinical trial drug for AD) were selected and used as template to identify essential pharmacophore features. The developed pharmacophores were subsequently used for database mining of "Zinc15" and "Asinex" databases. The similar pharmacophoric compounds obtained, were further subjected to various filters *i.e* drug-likeness property, *in-silico* BBB permeability and toxicity. Selected compounds were docked to identify the functional groups and fragments for key interactions with the targets (AChE and MMP-2).

4.1.2 Virtual screening of commercial and non-commercial databases

The purchasable subset of ZINC and ASINEX Databases were downloaded and structure-based virtual screening was performed against AChE and MMP-2 (PDB: 4EY7, 1HOV). Virtual screening was performed using Maestro 10.5.014 application (Schrödinger, LLC, USA, 2016-1). It involved three consecutive steps *viz*. receptor grid generation, ligand preparation and ligand docking. The proteins 4EY7, 1HOV were subjected to protein preparation and receptor gird generation with grid size of 10Å. Later, the ZINC and ASINEX database molecules were subjected to ligand preparation, followed by High-throughput virtual screening.

4.1.3 Extra precision molecular docking

The hit molecules obtained from off-target virtual screening and filtering steps (section 4.2) were further docked against AChE and MMP-2 (PDB: 4EY7, 1HOV) through Glide XP (extra precision) mode in Maestro, to study different ligand poses of a molecule and weed out the false positives to afford a better correlation between good score and good poses (352).

4.1.4 Molecular property, BBB permeability and toxicity prediction

Molecular properties *viz*. cLogP, cLogS, hydrogen bond donor, hydrogen bond acceptor and drug-likeness were analyzed according to Lipinski's rule of five, using OSIRIS DataWarrior. *In-silico* BBB permeability was performed by using ACD/Labs. Toxicity of molecules in the study *viz*. mutagenicity, tumorigenicity and irritancy were also predicted through OSIRIS DataWarrior (353).

4.1.5 Synthesis and characterization

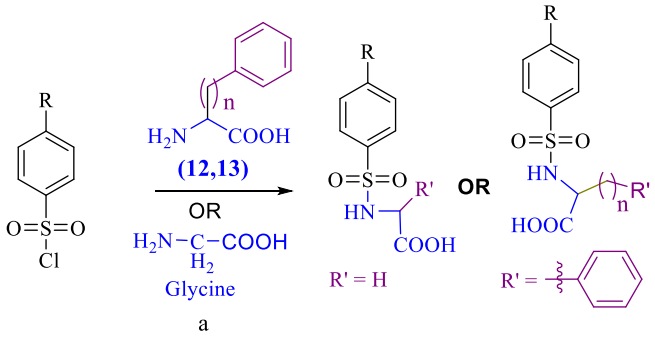
All the reagents were purchased from the Sigma-Aldrich, Avera, Spectrochem and Alfa Aesar. The progress of the reactions was monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254 plates (Merck KGaA) and ultraviolet light (254 nm) or iodine vapors for visualization of spots. Silica gel (60-120 mesh size) was used as adsorbent in column chromatography for the purification of the compounds. Melting points were determined by using automated melting point apparatus (Bamstead Electrothermal, UK). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Advance, 500 MHz spectrometer in DMSO-d_6 or $CDCl_3$. Chemical shift was measured in the ppm (δ) and coupling constant (*J*) was measured in Hz. Mass analysis was performed on LC-MS Spectrometer Model Q-ToF Micro Waters Mass spectra with EI

ion source and FTIR spectra was recorded on Bruker ALPHA-T (Germany) ATR /FT-IR instrument.

Purity of the compounds was determined by HPLC (Shimadzu LC 20 AD). Isocratic mobile phase was delivered by quaternary pumps with flow rate of 1ml/min. The mobile phase composition was phase A (acetonitrile) and phase B (methanol) in ratio of 1:9. 20 µL samples were injected into the HPLC column. SPD10AVP detector was used at 310 nm for the detection of the compounds. CLC C_{18} column (5µ, 25 cm x 4.6mm i.d, with CLC ODS, 4cm x 4.6 mm. i.d as guard column) was used for purification. The purity of the compounds was found to be above 98%.

4.1.5.1. Scheme I- Synthesis of aromatic piperazine derivatives

Step I: Synthesis of sulfonamides using different aminoacids



(1-13) **(11)** **(14-21)** **(22-35)**

Comp.	**1, 14,**	**2, 15,**	**3, 16,**	**4, 17,**	**5, 18,**	**6, 19,**	**7, 20,**	**8, 21,**	**9,**	**10,**	**11,**	**12,**	**13**
R	-4CH_3,	-4Cl,	-3Cl,	-4Br,	-4OCH_3,	-4NH_2,	-H,	-4F,	-4OH,	-2Cl,	-3OH,	-4NO_2,	-3Br

Comp.	**22, 31,**	**23, 32,**	**24, 33,**	**25, 34,**	**26, 35,**	**27,**	**28,**	**29,**	**30**
R	-4OH,	-4NO_2,	-4Br,	-3Br,	-4Cl,	-4NH_2,	-4CH_3,	-3CH_3,	-3OH
n	0,1,	0,1,	0,1,	0,1,	0,1,	0,	0,	0,	1

Reagents and conditions: (a) Na_2CO_3, Acetone, H_2O, stirring 24 h at rt.

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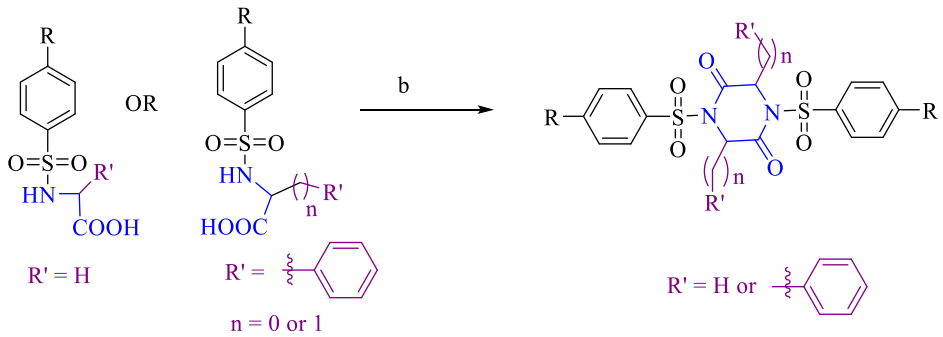
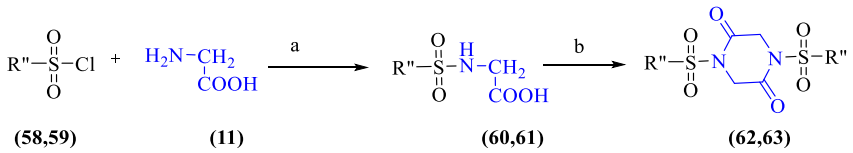
Step II: Cyclization of the sulfonamides into the piperazine ring



(14-21) **(22-35)** **(36-57)**

Reagents and conditions: (b) Ethyleneglycol, reflux 2-4 h at 120-150 ºC.

4.1.5.2. Scheme II: Aliphatic piperazine derivatives



Compound R"	60, -C_4H_9,	61 -C_3H_7

Reagents and conditions: (a) Na_2CO_3, acetone, H_2O, stirring 24 h at rt; (b) ethyleneglycol, refl ux 2-4 h at 120-150ºC.

4.1.5.3 General procedure for the synthesis of substituted sulfonamides using different aminoacids (14-21 and 22-35)

L-isomers of different amino acids were used for synthesis. The retention in configuration of the products was established by ^{1}H NMR spectra. Substituted aromatic or aliphatic sulfonylchlorides (1-13, 58, 59) (0.0143 mol, 1 equivalent) were dissolved in acetone (2 ml). The mixture was added to water (2 ml) containing glycine/phenylglycine/phenylalanine (0.0143 mol, 1 equivalent) and sodiumbicarbonate (0.0429 mol, 3 equivalent). Final reaction mixture was stirred at room temperature (rt) for 24 h and was monitored by TLC using ethylacetate and methanol (8:2) as the mobile phase. Acetone was evaporated under reduced pressure and reaction mixture was

neutralized with 1N HCl to get the product. The product was filtered and recrystallized with methanol to get the pure compounds(354).

2-((4-Hydroxyphenyl)sulfonamido)-2-phenylacetic acid **(22)**: It was synthesized as per general procedure earlier described in section 4.1.5.3 using 4-hydroxybenzenesulfonyl chloride (**9**) (2.8 g) to get compound **22** as white powder, yield 80%; mp 134-136 ºC; FTIR (KBr) cm^{-1}: 3480 (phenolic-OH-str.), 3400 (-NH-str.), 2400-2800 (-COOH-str.), 1161 (SO_2-str.); ^1H NMR (500 MHz, DMSO-d_6) δ 9.70 (s, 1H, -COOH), 7.81-7.76 (m, 2H, hydroxyphenyl-C_2, C_6), 7.68-7.62 (m, 2H, hydroxyphenyl-C_3, C_5), 7.40-7.25(m, 4H, phenyl-C_2, C_3, C_5, C_6), 7.24-7.18(m, 1H, phenyl-C_4), 5.22 (s, 1H, -OH), 4.82 (s, 1H, -CH), 4.31 (s, 1H, -NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.12 (-COOH), 161.42 (hydroxyphenyl-C_4), 137.16 (phenyl-C_1), 132.14 (hydroxyphenyl-C_1), 128.41 (hydroxyphenyl-C_2, C_6, phenyl-C_4), 127.70 (phenyl-C_2, C_6), 127.11 (hydroxyphenyl-C_3, C_5), 118.14 (phenyl-C_3, C_5), 64.41 (-CH); MS (ESI): *m/z* found 307.9[M^+]calculated for $C_{14}H_{13}NO_5S$ 307.0.

((3-Hydroxyphenyl)sulfonyl)phenylalanine **(30)**: Synthesized as per general procedure described earlier (section 4.1.5.3) using 3-hydroxybenzenesulfonyl chloride (**11**) (4.5 g) to get compound **30** as white powder, yield 73%; mp 162-163 ºC; FTIR (KBr) cm^{-1}: 3474 (phenolic-OH-str.), 3409 (-NH-str.), 2432-2842 (-COOH-str.), 1151 (SO_2-str.); ^1H NMR (500 MHz, DMSO-d_6) δ 9.51 (s, 1H, COOH), 7.39-7.38 (m, 2H, hydroxyphenyl-C_2, C_6), 7.37 (t, J = 5.5, 1H, hydroxyphenyl-C_5), 7.35-7.33 (m, 1H, hydroxyphenyl-C_4), 7.31 (t, J = 6.2, 2H, phenyl-C_3, C_5), 7.27 (d, J = 10.0, 2H, phenyl-C_2, C_6), 7.22 (t, J = 3.3, 1H, phenyl-C_4), 4.51 (t, J = 4.0, 1H, -CH), 3.8 (s, 1H, -NH), 3.31 (s, 1H, -OH), 2.84 (d, J = 0.9, 2H, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.14 (-COOH), 158.47 (hydroxyphenyl-C_1), 139.88 (hydroxyphenyl-C_3), 136.99 (phenyl-C_1), 129.14 (hydroxyphenyl-C_5), 128.84 (phenyl-C_2, C_6), 128.17 (phenyl-C_3, C_5), 127.54

(hydroxyphenyl-C_4), 123.49 (phenyl-C_4), 118.96 (hydroxyphenyl-C_6), 116.00 (hydroxyphenyl-C_2), 58.14 (-CH), 38.14 (-CH_2); MS (ESI): *m/z* found 321.7[M^+]calculated for $C_{15}H_{15}NO_5S$ 321.0.

((3-Bromophenyl)sulfonyl)phenylalanine **(34)**: It was synthesized as per the earlier mentioned general procedure (section 4.1.5.3) using 3-bromobenzenesulfonyl chloride (**13**) (5 g) to get compound **34** as brownish crystal, yield 82%; mp 151-153 ºC; FTIR (KBr) cm^{-1}: 3441 (-NH-str.), 2441-2814 (-COOH-str.), 1142 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.61 (s, 1H, COOH), 8.11 (s, 1H, bromophenyl-C_2), 7.76 (d, *J* = 10.3, 1H, bromophenyl-C_6), 7.64 (d, *J* = 2.9, 1H, bromophenyl-C_4), 7.20 (t, *J* = 5.8, 1H, bromophenyl-C_5), 7.16 (t, *J* = 6.0, 2H, phenyl-C_3, C_5), 7.14-7.7 (m, 3H, pheny-C_2, C_4, C_6), 5.41 (s, 1H, -NH), 4.47 (t, *J* = 4.3, 1H, -CH), 2.86 (d, *J* = 1.2, 2H, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.19 (bromophenyl-C_1), 142.14 (bromophenyl-C_3), 137.51 (bromophenyl-C_2), 135.25 (bromophenyl-C_4), 131.15 (phenyl-C_1), 130.84 (bromophenyl-C_5), 129.39 (phenyl-C_2, C_6), 129.13 (phenyl-C_3, C_5), 127.11 (phenyl-C_4), 124.25 (bromophenyl-C_6), 60.01 (-CH), 36.27 (-CH_2); MS (ESI): *m/z* found 383.3 [M^+] calculated for $C_{15}H_{14}BrNO_4S$ 382.9.

4.1.5.4 General procedure for synthesis of 1,4-Bis(substituted-phenylsulfonyl)3, 6 diphenyl/benzyl piperazine-2,5-dione derivatives (36-57 and 62, 63).

Compounds **14-35**, **60** and **61** (1.53 mmol, 1 equivalent) were treated with ethylene glycol (12.24 mmol, 8 equivalent) and heated up to 100 ºC to get the clear solution(355). The reaction mixture was refluxed at 120-150 ºC for 2-4 h. The final mixture was cooled and poured on crushed ice to get the solid product. The product was purified by column chromatography using 30% ethyl acetate in hexane as solvent over Silica gel (60-120 mesh).

1,4-Bis(phenylsulfonyl)piperazine-2,5-dione **(36)**: The compound was synthesized as per general procedure described in section 4.1.5.4 using (phenylsulfonyl)glycine (**20**) (0.33 g) to get compound **36** as white powder (reported by Koves G *et al.,*)(356), yield 40%; mp 293-294 ºC; FTIR (KBr) cm^{-1}: 3030 (Ar-str.), 1641 (C=O-str.), 1162 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.71(d, *J* = 8.5. 4H, phenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.27-7.26 (m, 6H, phenyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 4.24 (s, 4H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 157.71 (piperazine-C_2, C_5), 137.52 (phenyl-C_1 and $C_{1'}$), 133.31 (phenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 128.41 (phenyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 48.14 (piperazine-C_3, C_6); MS (ESI): *m/z* found 394.26 [M^+]; calculated for $C_{16}H_{14}N_2O_6S_2$ 394.42.

1,4-Ditosylpiperazine-2,5-dione **(37)**: It was synthesized as per general procedure described earlier (section 4.1.5.4) using tosylglycine (**14**) (0.35 g) to get compound **37** as white powder (reported by Berse C *et al.,*)(357), yield 36%; mp 281-283 ºC; FTIR (KBr) cm^{-1}: 3102 (Ar-str.), 1630 (C=O-str.), 1161 (SO_2-str.);^{1}H NMR (500 MHz, DMSO-d_6) δ 7.71-7.69 (m, 4H, ditosyl-C_2-C_6 and $C_{2'}$-$C_{6'}$), 7.64-7.61 (m, 4H, ditosyl-C_3-C_5 and $C_{3'}$-$C_{5'}$), 4.40 (s, 4H, piperazine-C_3, C_6), 2.30 (s, 6H, -CH_3, -CH_3);^{13}C NMR (125 MHz, DMSO-d_6) δ 159.64 (piperazine-C_2, C_5), 143.57(ditosyl-C_1 and $C_{1'}$), 139.67(ditosyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 131.41(ditosyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 127.97(ditosyl-C_4 and $C_{4'}$), 48.47 (piperazine-C_3, C_6), 22.41 (phenyl-CH_3); MS (ESI): *m/z* found 422.14 [M^+]; calculated for $C_{18}H_{18}N_2O_6S_2$ 422.47.

1,4-Bis((4-chlorophenyl)sulfonyl)piperazine-2,5-dione **(38)**: The compound was synthesized as per general procedure described in section 4.1.5.4 using ((4-chlorophenyl)sulfonyl)glycine (**15**) (0.38 g) to get compound **38** as white crystal, yield 37%; mp 162-165 ºC; FTIR (KBr) cm^{-1}: 2965 (Ar-str.), 1641 (C=O-str.), 1120 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.80 (d, *J* = 8.5, 4H, bis-chlorophenyl-C_2, C_6

and $C_{2'}$, $C_{6'}$), 7.72 (d, *J* = 7.7.0, 4H bis-chlorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 4.05 (s, 4H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 158.71(piperazine-C_2, C_5), 142.15 (bis-chlorophenyl-C_4, and $C_{4'}$), 139.45 (bis-chlorophenyl-C_1, and $C_{1'}$), 130.74 (bis-chlorophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 129.44 (bis-chlorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 48.10 (piperazine-C_3, C_6); MS (ESI): *m/z* found 463.4, 465.2, 467.8 [M^+, M^{+2}, M^{+4} (9:6:1)]; calculated for $C_{16}H_{12}Cl_2N_2O_6S_2$ 463.3.

1,4-Bis((3-chlorophenyl)sulfonyl)piperazine-2,5-dione **(39)**: It was synthesized as per general procedure described in section 4.1.5.4 using ((3-chlorophenyl)sulfonyl)glycine (**16**) (0.38 g) to get compound **39** as white crystal, yield 35%; mp 141-142 ºC; FTIR (KBr) cm^{-1}: 2962 (Ar-str.), 1652 (C=O-str.), 1132 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.03 (s, 2H, bis-chlorophenyl-C_2, $C_{2'}$), 7.89-7.75 (m, 2H, bis-chlorophenyl-C_6, $C_{6'}$), 7.68-7.55 (m, 2H, bis-chlorophenyl-C_4, $C_{4'}$), 7.47-7.43 (m, 2H, bis-chlorophenyl-C_5, $C_{5'}$), 4.51 (s, 4H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.41 (piperazine-C_2, C_5), 139.71 (bis-chlorophenyl-C_3, and $C_{3'}$), 132.51 (bis-chlorophenyl-C_1, and $C_{1'}$), 131.95 (bis-chlorophenyl-C_2 and $C_{2'}$), 130.15 (bis-chlorophenyl-C_4, and $C_{4'}$), 129.31 (bis-chlorophenyl-C_6, and $C_{6'}$), 127.47 (bis-chlorophenyl-C_5, and $C_{5'}$), 49.15 (piperazine-C_3, C_6); MS (ESI): *m/z* found 463.2, 465.4, 467.1 [M^+, M^{+2}, M^{+4} (9:6:1)]; calculated for $C_{16}H_{12}Cl_2N_2O_6S_2$ 463.3.

1,4-Bis((4-bromophenyl)sulfonyl)piperazine-2,5-dione **(40)**: This compound was also synthesized as per general procedure mentioned in section 4.1.5.4 using ((4-bromophenyl)sulfonyl)glycine (**17**) (0.45 g) to get compound **40** as pale brown crystal, yield 35%; mp 161-164 ºC; FTIR (KBr) cm^{-1}: 3014 (Ar-str.), 1641 (C=O-str.), 1142 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.74 (d, *J* = 8.7, 4H, bis-bromophenyl-C_2, C_6 and C_2', C_6'), 7.64 (d, *J* = 8.0, 4H bis-bromophenyl-C_3, C_5 and C_3', C_5'), 4.00 (s, 4H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.51 (piperazine-C_2, C_5),

138.84 (bis-bromophenyl-C_4, and $C_{4'}$), 132.10 (bis-bromophenyl-C_1, and $C_{1'}$), 130.24 (bis-bromophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 129.61 (bis-bromophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 49.27 (piperazine-C_3, C_6); MS (ESI): *m/z* found 551.4, 553.25, 555.1 [M^+, M^{+2}, M^{+4} (1:2:1)]; calculated for $C_{16}H_{12}Br_2N_2O_6S_2$ 551.8.

1,4-Bis((4-methoxyphenyl)sulfonyl)piperazine-2,5-dione **(41)**: Synthesized as per general procedure described earlier (section 4.1.5.4) using ((4-methoxyphenyl)sulfonyl)glycine (**18**) (0.37 g) to get compound **41** as white powder, yield 31%; mp 162-165 ºC; FTIR (KBr) cm^{-1}: 3042 (Ar-str.), 1645 (C=O-str.), 1127 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.76-7.74 (m, 4H, bis-methoxyphenyl-C_2-C_6 and $C_{2'}$-$C_{6'}$), 7.68-7.65 (m, 4H, bis-methoxyphenyl-C_3-C_5 and $C_{3'}$-$C_{5'}$), 4.42 (s, 4H, piperazine-C_3, C_6), 2.80 (s, 6H, -OCH_3, -OCH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.84 (piperazine-C_2, C_5), 157.51 (bis-methoxyphenyl-C_4, and $C_{4'}$), 130.21 (bis-methoxyphenyl-C_1, and $C_{1'}$), 129.10 (bis-methoxyphenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 112.64 (bis-methoxyphenyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 55.41 (-OCH_3), 47.27 (piperazine-C_3, C_6); MS (ESI): *m/z* found 454.8 [M^+]; calculated for $C_{18}H_{18}N_2O_8S_2$ 454.4.

1,4-Bis((4-aminophenyl)sulfonyl)piperazine-2,5-dione **(42)**: The compound was synthesized as per general procedure mentioned in section 4.1.5.4 using ((4-aminophenyl)sulfonyl)glycine (**19**) (0.35 g) to get compound **42** as light brown powder, yield 34%; mp 155-157 ºC; FTIR (KBr) cm^{-1}: 3331, 3291 (NH_2-str.), 2957 (Ar-str.), 1651 (C=O-str.), 1131 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.54 (d, J = 8.5, 4H, bis-aminophenyl-C_2-C_6 and $C_{2'}$-$C_{6'}$), 7.38 (d, J = 7.8, 4H, bis-aminophenyl-C_3-C_5 and $C_{3'}$-$C_{5'}$), 4.20 (s, 4H, piperazine-C_3, C_6), 1.50 (s, 4H, -NH_2, -NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.74 (piperazine-C_2, C_5), 154.34 (bis-aminophenyl-C_4, and $C_{4'}$), 130.14 (bis-aminophenyl-C_1, and $C_{1'}$), 126.94 (bis-aminophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$),

115.24 (bis-aminophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 48.41 (piperazine-C_3, C_6); MS (ESI): *m/z* found 424.2 [M^+]; calculated for $C_{16}H_{16}N_4O_6S_2$ 424.4.

1,4-Bis((4-fluorophenyl)sulfonyl)piperazine-2,5-dione **(43)**: Synthesized as per general procedure described in section 4.1.5.4 using ((4-fluorophenyl)sulfonyl)glycine (**21**) (0.36 g) to get compound **43** as white crystal, yield 36%; mp 182-184 ºC; FTIR (KBr) cm^{-1}: 3014 (Ar-str.), 1625 (C=O-str.), 1135 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.10 (d, *J* = 8.3, 4H bis-chlorophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.80 (d, *J* = 7.3, 4H, bis-fluorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 4.20 (s, 4H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.21 (piperazine-C_2, C_5), 159.61 (fluorophenyl-C_4 and $C_{4'}$), 136.14 (fluorophenyl-C_1 and $C_{1'}$), 132.44 (fluorophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 118.57 (fluorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 49.41 (piperazine-C_3, C_6); MS (ESI): *m/z* found 430.2 [M^+]; calculated for $C_{16}H_{12}F_2N_2O_6S_2$ 430.4.

1,4-Bis((4-hydroxyphenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(44)**: It was also synthesized as per general procedure mentioned in section 4.1.5.4 using 2-((4-hydroxyphenyl)sulfonamido)-2-phenylacetic acid (**22**) (0.50 g) to get compound **44** as white powder, yield 33%; mp 185-187 ºC; FTIR (KBr) cm^{-1}: 3379 (-OH-str.), 2895 (Ar-str.), 1662 (C=O-str.), 1169 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.79-7.77 (m, 4H, bis-hydroxyphenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.72-7.56 (m, 4H, bis-hydroxyphenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.48-7.06 (m, 10H, dipheny), 5.30 (s, 2H, piperazine-C_3, C_6), 4.18 (s, 2H, -OH, -OH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.71(piperazine-C_2, C_5), 162.51(bis-hydroxyphenyl-C_4, and $C_{4'}$), 136.19 (bis-hydroxyphenyl-C_1, and $C_{1'}$), 129.71(bis-hydroxyphenyl-C_2, and $C_{6'}$), 128.91(bis- hydroxyphenyl-C_3, and $C_{5'}$), 128.31(dibenzyl-C_1 and $C_{1'}$), 127.84 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 127.51(dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$,$C_{5'}$), 117.81(dibenzyl-C_4 and $C_{4'}$), 69.10 (piperazine-C_3, C_6); MS (ESI): *m/z* found 578.5 [M^+]; calculated for $C_{28}H_{22}N_2O_8S_2$ 578.6.

1,4-Bis((4-aminophenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(45)**: Synthesized as per general procedure mentioned earlier (section 4.1.5.4) using 2-((4-aminophenyl)sulfonamido)-2-phenylacetic acid (**27**) (0.45 g) to get compound **45** as light brown powder, yield 33%; mp 174-175 ºC; FTIR (KBr) cm^{-1}: 3321, 3284 (NH_2-str.), 2937 (Ar-str.), 1651 (C=O-str.), 1120 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.74-7.71 (m, 4H, bis-aminophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.62-7.45 (m, 4H, bis-aminophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.39-6.80 (m, 10H, dipheny), 5.21 (s, 2H, piperazine-C_3, C_6), 4.10 (s, 4H, -NH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.75 (piperazine-C_2, C_5), 154.47 (bis-aminophenyl-C_4, and $C_{4'}$), 136.01 (bis-aminophenyl-C_1, and $C_{1'}$), 131.64 (bis-aminophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 130.84 (bis-aminophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 129.87 (dibenzyl-C_1 and $C_{1'}$), 128.57 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 126.37 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 115.14 (dibenzyl-C_4 and $C_{4'}$), 69.47 (piperazine-C_3, C_6); MS (ESI): *m/z* found 576.2 [M^+]; calculated for $C_{28}H_{24}N_4O_6S_2$ 576.6.

1,4-Bis((4-chlorophenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(46)**: This was synthesized as per general procedure described in section 4.1.5.4 using 2-((4-chlorophenyl)sulfonamido)-2-phenylacetic acid (**26**) (0.50 g) to get compound **46** as white crystal, yield 32%; mp 181-183 ºC; FTIR (KBr) cm^{-1}: 3014 (Ar-str.), 1647 (C=O-str.), 1124 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.82-7.79 (m, 4H, bis-chlorophenyl-C_2, C_6 and C_2', C_6'), 7.78-7.60 (m, 4H, bis-chlorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.35-7.07 (m, 10H, dipheny), 5.35 (s, 2H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.21 (piperazine-C_2, C_5), 139.78 (bis-chlorophenyl-C_4, and $C_{4'}$), 137.97 (bis-chlorophenyl-C_1, and $C_{1'}$), 129.68 (bis-chlorophenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 128.855 (bis-chlorophenyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 119.47 (dibenzyl-C_1 and $C_{1'}$), 118.62 (dibenzyl-C_2- C_6 and C_2- $C_{6'}$), 67.34 (piperazine-C_3, C_6); MS (ESI): *m/z* found 615.1, 617.2, 619.4 [M^+, M^{+2}, M^{+4} (9:6:1)]; calculated for $C_{28}H_{20}Cl_2N_2O_6S_2$ 615.5.

1,4-Bis((4-nitrophenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(47)**: Synthesized as per general procedure described earlier (section 4.1.5.4) using 2-((4-nitrophenyl)sulfonamido)-2-phenylacetic acid (**23**) (0.51 g) to get compound **47** as pale yellow crystal, yield 39%; mp 224-227 ºC; FTIR (KBr) cm^{-1}: 3067 (Ar-str.), 1669 (C=O-str.), 1150 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.43 (d, *J* = 7.2, 4H, bis-nitrophenyl- C_3, C_5 and $C_{3'}$, $C_{5'}$), 8.22 (d, *J* = 8.4, 4H, bis-nitrophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.52-7.08 (m, 10H, dipheny), 5.52 (s, 2H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 165.97 (piperazine-C_2, C_5), 154.12 (bis-nitrophenyl-C_4 and $C_{4'}$), 145.02 (bis-nitrophenyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 137.12 (bis-nitrophenyl-C_1 and $C_{1'}$), 130.86 (bis-nitrophenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 129.27 (dibenzyl-C_1 and $C_{1'}$), 127.47 (dibenzyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 124.41 (dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 68.17 (piperazine-C_3, C_6); MS (ESI): *m/z* found 636.2 [M^+]; calculated for $C_{28}H_{20}N_4O_{10}S_2$ 636.6.

3,6-Diphenyl-1,4-ditosylpiperazine-2,5-dione **(48)**: Synthesized as per general procedure mentioned in section 4.1.5.4 using 2-((4-methylphenyl)sulfonamido)-2-phenylacetic acid (**28**) (0.47 g) to get compound **48** as white powder, yield 38%; mp 138-140 ºC; FTIR (KBr) cm^{-1}: 3142 (Ar-str.), 1652(C=O-str.), 1142 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.74 (d, *J* = 8.0, 4H, ditosyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.67 (d, J = 7.6, 4H, ditosyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.43-7.01 (m, 10H, dipheny), 5.25 (s, 2H, piperazine-C_3, C_6), 2.18 (s, 6H, -CH_3, -CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.57 (piperazine-C_2, C_5), 141.84 (ditosyl-C_1 and $C_{1'}$), 137.72 (ditosyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 134.24 (ditosyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 129.84 (ditosyl-C_4 and $C_{4'}$), 129.10 (dibenzyl-C_1 and $C_{1'}$), 128.31 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 127.17 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 126.4 (dibenzyl-C_4 and $C_{4'}$), 69.10 (piperazine-C_3, C_6), 20.41 (-CH_3); MS (ESI): *m/z* found 574.1 [M^+]; calculated for $C_{30}H_{26}N_2O_6S_2$ 574.6.

3,6-Diphenyl-1,4-bis(m-tolylsulfonyl)piperazine-2,5-dione **(49)**: It was synthesized as per general procedure earlier described in section 4.1.5.4 using 2-((3-methylphenyl)sulfonamido)-2-phenylacetic acid (**29**) (0.47 g) to get compound **49** as white powder, yield 37%; mp 152-154 ºC; FTIR (KBr) cm^{-1}: 2951 (Ar-str.), 1614 (C=O-str.), 1146 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.93 (s, 2H, bis-tolyl-C_2, and $C_{2'}$), 7.79 (d, *J* = 8.3, 2H, bis-tolyl-C_6, and $C_{6'}$), 7.58-7.33 (m, 4H, bis-tolyl-C_4, C_5 and $C_{4'}$, $C_{5'}$), 7.23 (d, *J* = 8.0, 4H, dipheny-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.20-6.53 (m, 6H, dipheny-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 4.24 (s, 2H, piperazine-C_3, C_6), 2.00 (s, 6H, -CH_3, -CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.24 (piperazine-C_2, C_5), 137.61 (ditosyl-C_1 and $C_{1'}$), 135.41 (ditosyl-C_6 and $C_{6'}$), 133.41 (ditosyl-C_2 and $C_{2'}$), 129.31 (ditosyl-C_5 and $C_{5'}$), 128.71 (ditosyl-C_4 and $C_{4'}$), 127.74 (ditosyl-C_3 and $C_{3'}$), 127.31 (dibenzyl-C_1 and $C_{1'}$), 126.74 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 125.46 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 123.71 (dibenzyl-C_4 and $C_{4'}$), 69.17 (piperazine-C_3, C_6), 21.41 (-CH_3); MS (ESI): *m/z* found 574.2 [M^+]; calculated for $C_{30}H_{26}N_2O_6S_2$ 574.6.

1,4-Bis((4-bromophenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(50)**: The compound was synthesized as per general procedure mentioned in section 4.1.5.4 using 2-((4-bromophenyl)sulfonamido)-2-phenylacetic acid (**24**) (0.57 g) to get compound **50** as light brown crystal, yield 31%; mp 181-184 ºC; FTIR (KBr) cm^{-1}: 3014 (Ar-str.), 1625 (C=O-str.), 1142 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.79 (d, *J* = 8.6, 4H, bis-bromophenyl-C_2, C_6 and C_2', C_6'), 7.73 (d, *J* = 7.3, 4H, bis-bromophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.47-7.04 (m, 10H, dipheny), 4.75 (s, 2H, piperazine-C3, C6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.66 (piperazine-C_2, C_5), 139.62 (bromophenyl-C_1 and $C_{1'}$), 135.11 (bromophenyl-C_4 and $C_{4'}$), 132.14 (bromophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 131.01 (bromophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 130.77 (dibenzyl-C_1 and $C_{1'}$), 130.42 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 129.35 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 127.46 (dibenzyl-C_4 and $C_{4'}$),

69.17 (piperazine-C_3, C_6); MS (ESI): *m/z* found 704.1, 706.4, 708.3 [M^+, M^{+2}, M^{+4}(1:2:1)]; calculated for $C_{28}H_{20}Br_2N_2O_6S_2$ 704.4.

1,4-Bis((3-bromophenyl)sulfonyl)-3,6-diphenylpiperazine-2,5-dione **(51)**: Synthesized as per general procedure given in section 4.1.5.4 using 2-((3-bromophenyl)sulfonamido)-2-phenylacetic acid (**25**) (0.57 g) to get compound **51** as light brown crystal, yield 30%; mp 190-192 ºC; FTIR (KBr) cm^{-1}: 3010 (Ar-str.), 1621 (C=O-str.), 1131 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.96 (s, 2H, bis-bromophenyl-C_2, $C_{2'}$), 7.83 (d, *J* = 8.1, 2H, bis-bromophenyl-C_6, $C_{6'}$), 7.63 (d, *J* = 5.2, 2H, bis-bromophenyl-C_4, $C_{4'}$), 7.41 (t, *J* = 7.5, 2H, bis-bromophenyl-C_5, $C_{5'}$), 7.45-7.01 (m, 10H, dipheny), 4.76 (s, 2H, piperazine-C_3, C_6); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.47 (piperazine-C_2, C_5), 141.41 (bromophenyl-C_3 and $C_{3'}$), 136.51 (bromophenyl-C_1 and $C_{1'}$), 135.02 (bromophenyl-C_2 and $C_{2'}$), 132.11(bromophenyl-C_6 and $C_{6'}$), 130.74 (bromophenyl-C_4 and $C_{4'}$), 129.35 (bromophenyl-C_5 and $C_{5'}$), 127.44 (dibenzyl-C_1 and $C_{1'}$), 125.91 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 123.87 (dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 69.19 (piperazine-C_3, C_6); MS (ESI): *m/z* found 704.2, 706.2, 708.7 [M^+, M^{+2}, M^{+4}(1:2:1)]; calculated for $C_{28}H_{20}Br_2N_2O_6S_2$ 704.4.

3,6-Dibenzyl-1,4-bis((4-chlorophenyl)sulfonyl)piperazine-2,5-dione **(52)**: The compound was synthesized as per general procedure (mentioned in section 4.1.5.4) using ((4-chlorophenyl)sulfonyl)phenylalanine (**35**) (0.53 g) to get compound **52** as white crystal, yield 32%; mp 177-180 ºC; FTIR (KBr) cm^{-1}: 3027 (Ar-str.), 1621 (C=O-str.), 1141 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.80-7.78 (m, 4H, bis-chlorophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.78-7.62 (m, 4H, bis-chlorophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.45-7.03 (m, 10H, dibenzyl), 4.64-4.63 (m, 2H, piperazine-C_3, C_6), 3.00 (dd, *J* = 6.6, 4H, -CH_2, -CH_2);^{13}C NMR (125 MHz, DMSO-d_6) δ 164.51 (piperazine-C_2, C_5), 140.75 (bis-chlorophenyl-C_4, and $C_{4'}$), 138.62 (bis-chlorophenyl-C_1, and $C_{1'}$), 136.72

(bis-chlorophenyl-C_2, and $C_{6'}$), 131.64 (bis-chlorophenyl-C_3, and $C_{5'}$), 128.71 (dibenzyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 122.00 (dibenzyl-C_1 and $C_{1'}$), 120.62 (dibenzyl-C_2-C_6 and $C_{2'}$- $C_{6'}$), 61.72 (piperazine-C_3, C_6), 30.05 (benzyl-CH_2); MS (ESI): *m/z* found 643.2, 645.1, 647.4 [M^+: M^{+2}: M^{+4} (9:6:1)]; calculated for $C_{30}H_{24}Cl_2N_2O_6S_2$ 643.5.

3,6-Dibenzyl-1,4-bis((4-hydroxyphenyl)sulfonyl)piperazine-2,5-dione **(53)**: Synthesized as per general procedure described in section 4.1.5.4 using ((4-hydroxyphenyl)sulfonyl)phenylalanine (**31**) (0.49 g) to get compound **53** as white powder, yield 35%; mp 181-183 ºC; FTIR (KBr) cm^{-1}: 3450 (-OH-str.), 2981 (Ar-str.), 1651 (C=O-str.), 1142 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.76 (d, *J* = 8.5, 4H, bis-hydroxyphenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.70 (d, *J* = 7.4, 4H, bis-hydroxyphenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.38-7.12 (m, 4H, dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.10-6.80 (m, 6H, dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 4.50 (t, *J* = 6.3, 2H, piperazine-C_3, C_6), 4.02 (s, 2H, -OH, -OH), 3.08 (d, 4H, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.51(piperazine-C_2, C_5), 161.79 (bis-hydroxyphenyl-C_4, and $C_{4'}$), 137.75 (bis-hydroxyphenyl-C_1, and $C_{1'}$), 128.47 (bis-hydroxyphenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 129.19 (bis-hydroxyphenyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 128.31 (dibenzyl-C_1 and $C_{1'}$), 127.82 (dibenzyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 127.31 (dibenzyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 116.78 (dibenzyl-C_4 and $C_{4'}$), 63.27 (piperazine-C_3, C_6), 37.17 (benzyl-CH_2); MS (ESI): *m/z* found 606.2 [M^+]; calculated for $C_{30}H_{26}N_2O_8S_2$ 606.6.

3,6-Dibenzyl-1,4-bis((3-hydroxyphenyl)sulfonyl)piperazine-2,5-dione **(54)**: Synthesized as per general procedure mentioned in section 4.1.5.4 using ((3-hydroxyphenyl)sulfonyl)phenylalanine (**30**) (0.49 g) to get compound **54** as white powder, yield 31%; mp 175-177 ºC; FTIR (KBr) cm^{-1}: 3431 (-OH-str.), 3171 (Ar-str.), 1661 (C=O-str.), 1131 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.98 (s, 2H, bis-hydroxyphenyl-C_2, $C_{2'}$), 7.84 (d, *J* = 8.0, 2H, bis-hydroxyphenyl-C_6, $C_{6'}$), 7.64 (d, *J* =

5.5, 2H, bis-hydroxyphenyl-$C_{4,}$ $C_{4'}$), 7.43 (t, *J* = 7.5, 2H, bis-hydroxyphenyl-$C_{5,}$ $C_{5'}$), 7.34 (t, *J* = 7.2, 4H, dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.32-6.76 (m, 6H, dibenzyl-C_2, C_4, C_6 and $C_{2'}$, $C_{4'}$ $C_{6'}$), 4.45 (t, *J* = 6.5, 2H, piperazine-C_3, C_6), 3.80 (s, 2H, -OH, -OH), 3.10-3.87 (m, 4H, -CH_2, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.51 (piperazine-C_2, C_5), 157.71 (bis-hydroxyphenyl-C_3, and $C_{3'}$), 137.51 (bis-hydroxyphenyl-C_1, and $C_{1'}$), 136.54 (bis-hydroxyphenyl-C_2, and $C_{2'}$), 129.71(bis-hydroxyphenyl-C_6, and $C_{6'}$), 128.18 (bis-hydroxyphenyl-C_4, and $C_{4'}$), 127.21 (bis-hydroxyphenyl-C_5, and $C_{5'}$), 126.45 (dibenzyl-C_1 and $C_{1'}$), 124.14 (dibenzyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 119.42 (dibenzyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 115.28 (dibenzyl-C_4 and $C_{4'}$), 63.47 (piperazine-C_3, C_6), 37.16 (benzyl-CH_2); MS (ESI): *m/z* found 606.1 [M^+]; calculated for $C_{30}H_{26}N_2O_8S_2$ 606.6.

3,6-Dibenzyl-1,4-bis((4-nitrophenyl)sulfonyl)piperazine-2,5-dione **(55)**: Synthesized as per general procedure given in section 4.1.5.4 using ((4-nitrophenyl)sulfonyl)phenylalanine (**32**) (0.53 g) to get compound **55** as pale yellow crystal, yield 40%; mp 213-214 ºC; FTIR (KBr) cm^{-1}: 3087 (Ar-str.), 1657 (C=O-str.), 1145 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.40 (d, *J* = 9.0, 4H, bis-nitrophenyl-C_2, C_6 and $C_{2',}$ $C_{6'}$), 8.20 (d, *J* = 5.1, 4H, bis-nitrophenyl- C_3, C_5 and $C_{3',}$ $C_{5'}$), 7.51 (d, *J* = 8.4, 4H, dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.47 (t, *J* = 7.3, 4H, dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.30 (t, *J* = 6.8, 2H, dibenzyl-C_4 and $C_{4'}$), 4.68 (t, *J* = 3.1, 2H, piperazine-C_3, C_6), 3.15-3.01 (m, 4H, -CH_2, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 165.71 (piperazine-C_2, C_5), 154.24 (bis-nitrophenyl-C_4 and $C_{4'}$), 145.15 (bis-nitrophenyl-C_3, C_5 and $C_{3'}$ $C_{5'}$), 138.72 (bis-nitrophenyl-C_1 and $C_{1'}$), 129.71 (bis-nitrophenyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 129.08 (dibenzyl-C_1 and $C_{1'}$), 127.72 (dibenzyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 126.17 (dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$, $C_{5'}$), 63.75 (piperazine-C_3, C_6), 37.14 (benzyl-CH_2); MS (ESI): *m/z* found 664.4 [M^+]; calculated for $C_{30}H_{24}N_4O_{10}S_2$ 664.6.

3,6-Dibenzyl-1,4-bis((4-bromophenyl)sulfonyl)piperazine-2,5-dione **(56)**: This was also synthesized as per general procedure earlier mentioned (section 4.1.5.4) using ((4-bromophenyl)sulfonyl)phenylalanine (**33**) (0.59 g) to get compound **56** as brownish crystal, yield 32%; mp 167-169 ºC; FTIR (KBr) cm^{-1}: 3015 (Ar-str.), 1661 (C=O-str.), 1109 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.79 (d, *J* = 8.5, 4H, bis-bromophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.78 (d, *J* = 7.5, 4H, bis-bromophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 7.35 (d, *J* = 8.6, 4H, dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.33-7.05 (m, 6H, dibenzyl-C_3, C_4, C_5 and $C_{2'}$, $C_{4'}$, $C_{5'}$), 4.44 (t, *J* = 3.5, 2H, piperazine-C_3, C_6), 3.01-2.90 (m, 4H, -CH_2, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.74 (piperazine-C_2, C_5), 139.71 (bis-bromophenyl-C_4 and $C_{4'}$), 137.58 (bis-bromophenyl-C_1 and $C_{1'}$), 132.21(bis-bromophenyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 131.10 (bis-bromophenyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 130.37 (dibenzyl-C_1 and $C_{1'}$), 129.75 (dibenzyl-C_2, C_6 and $C_{2'}$ $C_{6'}$), 128.97 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 127.61 (dibenzyl-C_4 and $C_{4'}$), 62.31 (piperazine-C_3, C_6), 37.47 (benzyl-CH_2); MS (ESI): *m/z* found 732.3, 734.2, 736.2 [M^+, M^{+2}, M^{+4} (1:2:1)]; calculated for $C_{30}H_{24}Br_2N_2O_6S_2$ 732.4.

3,6-Dibenzyl-1,4-bis((3-bromophenyl)sulfonyl)piperazine-2,5-dione **(57)**: Synthesized as per general procedure mentioned in section 4.1.5.4 using ((3-bromophenyl)sulfonyl)phenylalanine (**34**) (0.59 g) to get compound **57** as brownish crystal, yield 31%; mp 152-154 ºC; FTIR (KBr) cm^{-1}: 2984 (Ar-str.), 1645 (C=O-str.), 1114 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.99 (s, 2H, bis-bromophenyl-C_2, and $C_{2'}$), 7.86 (s, 2H, bis-bromophenyl-C_6, $C_{6'}$), 7.66 (d, *J* = 6.5, 2H, bis-bromophenyl-C_4, $C_{4'}$), 7.45 (t, *J* = 7.7, 2H, bis-bromophenyl-C_5, $C_{5'}$), 7.36 (d, *J* = 8.0, 4H, dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 7.33-7.30 (m, 6H, dibenzyl-C_3, C_4, C_5 and $C_{3'}$, $C_{4'}$ $C_{5'}$), 4.48 (t, *J* = 6.0, 2H, piperazine-C_3, C_6), 3.10-3.00 (m, 4H, -CH_2, -CH_2); ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.74 (piperazine-C_2, C_5), 141.51 (bis-bromophenyl-C_3 and $C_{3'}$), 137.74 (bis-

bromophenyl-C_1 and $C_{1'}$), 136.61 (bis-bromophenyl-C_2 and $C_{2'}$), 132.10 (bis-bromophenyl-C_6 and $C_{6'}$), 130.41(bis-bromophenyl-C_4 and $C_{4'}$), 129.76 (bis-bromophenyl-C_5 and $C_{5'}$), 129.21 (dibenzyl-C_1 and $C_{1'}$), 127.52 (dibenzyl-C_2, C_6 and $C_{2'}$, $C_{6'}$), 125.14 (dibenzyl-C_3, C_5 and $C_{3'}$, $C_{5'}$), 123.71 (dibenzyl-C_4 and $C_{4'}$), 62.24 (piperazine-C_3, C_6), 37.21 (benzyl-CH_2); MS (ESI): *m/z* found 732.1, 734.2, 736.3 [M^+, M^{+2}, M^{+4}(1:2:1)]; calculated for $C_{30}H_{24}Br_2N_2O_6S_2$ 732.4.

1,4-Bis(butylsulfonyl)piperazine-2,5-dione **(62)**: It was synthesized as per general procedure given in section 4.1.5.4 using (butylsulfonyl)glycine (**60**) (0.30 g) to get compound **62** as brownish powder, yield 40 %; mp 124-126 ºC; FTIR (KBr) cm^{-1}: 1594 (C=O-str.), 1078 (SO_2-str.); ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.49 (s, 4H, piperazine-C_3, C_6), 4.41 (t, *J* = 6.9, 4H, -CH_2), 3.61 (m, 4H, -CH_2, -CH_2), 3.00 (m, 4H, CH_2), 1.01 (t, *J* = 7.2, 6H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 156.74 (piperazine-C_2, C_5), 51.54 (piperazine-C_3, C_6), 45.34 (-CH_2), 27.52 (-CH_2), 21.11 (-CH_2), 14.14 (-CH_3); MS (ESI): *m/z* found 354.3 [M^+]; calculated for $C_{12}H_{22}N_2O_6S_2$ 354.4.

1,4-Bis(propylsulfonyl)piperazine-2,5-dione **(63)**: Synthesized as per general procedure described in section 4.1.5.4 using (propylsulfonyl)glycine (**61**) (0.28 g) to get compound **63** as brownish powder, yield 40 %; mp 130-131 ºC; FTIR (KBr) cm^{-1}: 1597 (C=O-str.), 1073 (SO_2-str.);^{1}H NMR (500 MHz, DMSO-d_6) δ 4.51 (s, 4H, piperazine-C_3, C_6), 3.71 (t, *J* = 6.5, 4H, -CH_2, -CH_2), 2.50 (m, 4H, CH_2), 1.00 (t, *J* = 7.0, 6H, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6) δ 157.64 (piperazine-C_2, C_5), 55.40 (piperazine-C_3, C_6), 46.41 (-CH_2), 18.71 (-CH_2), 13.18(-CH_3); MS (ESI): *m/z* found 326.1 [M^+]; calculated for $C_{10}H_{18}N_2O_6S_2$ 326.3.

4.1.6 Biological profiling

4.1.6.1 *In-vitro* AChE and BuChE inhibition assays

The procedure described by Ellman *et al.* was used for AChE and BuChE inhibition assays with minor modifications(358). AChE and BuChE were purchased from sigma Aldrich (CAS No.9000-81-1, 9001-08-5 respectively). Acetylthiocholine iodide (ATCI), Butyrylthiocholine iodide (BTCI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB-Ellman's reagent) were purchased from Himedia. The assays were performed in tris-HCl buffer (pH 8) and donepezil was used as standard compound. The percentage inhibitions were determined at 100 µM and 50 µM for the selection of concentration range for IC_{50} assays. Six different concentrations of 200 nM, 100 nM, 50 nM, 25 nM, 1 nM and 0.1 nM of test compounds were used to determine the IC_{50}. 50 µL of AChE (1.00 U mL^{-1}) or 50 µL of BuChE (0.6 U mL^{-1}) and 20 µL of test or standard compounds were incubated in 96 well plate for 30 min. at rt. 100 µL (1.5 mM) of DTNB was added in the above solution. The substrate *i.e.*ATCI (15 mM, 10 µL) or BTCI (30 mM, 10 µL) was added into it and absorbance was recorded immediately at 415 nm for 20 min. at 1 min. interval using Synergy HTX multi-mode reader (BioTek, USA). The IC_{50} values were calculated using absorbance obtained from the test and standard compounds. The assays were performed in triplicate and in three independent runs.

4.1.6.2 AChE enzyme kinetics

Enzyme (AChE) kinetics study was performed to understand the mechanism of enzyme action of compounds **46**. Seven different substrate concentrations in the range of 20-70 nM were used in the kinetic study. Compound **46** was used in three concentrations in the range of 10-40 nM. Each concentration of the compound **46** was used with seven different concentrations of the substrate. The activity was measured for 10 min at an

interval of 2 min in absence and presence of compound **46**. The product formed during time frame of 10 min was calculated by Beer-Lambert law. The velocity of the enzyme reaction was obtained by plotting the 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 5(359). Ki value was determined by Dixon method in which slope of the lines from double reciprocal Lineweaver-Burk plot was plotted as a function of test compounds **46** (360). The enzyme kinetic assay was performed in triplicate.

4.1.6.3 *In-vitro* MMP-2 inhibition assay

MMP-2 inhibitor screening assay kit (Fluorometric) was purchased from Abcam (ab139447). Seven different concentrations (0.1-1000 nM) of the test compounds were used in enzyme inhibition studies. MMP-2 enzyme (20 µL) was added to each well of the plate in final concentration of 1.16 U. The test compounds in seven different concentrations (20 µL) were added to enzyme and was followed by incubation of the plate for 60 min. to allow the interaction of enzyme-test compounds. Fluorogenic substrate in the final concentration of 4 µM (10 µL) was added to the incubated mixture and volume was made to 100 µL by fluorogenic assay buffer. Ex/Em: 328/420 was used to record the fluorescence spectra in a microplate (HTX multi-mode reader, BioTek, USA) at 1 min interval for 10 min. Assay was performed in triplicate with calibration sample containing 10 µL (4 µM) of the substrate and 90 µL of assay buffer, control without inhibitor and inhibitor containing 20 µL (6.5 µM) of *N*-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH). The activity was expressed in relative fluorescent units (RFU) and the control reaction was used to calculate

percentage inhibition. The data was processed by using GraphPad Prism 5, Excel and IC_{50} were calculated by using the formula- $Vi/Vo = 1/1+([I]/IC_{50})$

Where *Vi* is initial velocity of product formation in presence of inhibitor Vo and I is the initial velocity in the absence of inhibitor I.

4.1.6.4 MMP-2 enzyme kinetics

Kinetic study was performed for the determination of mechanism of enzyme inhibition. Five different concentrations of substrate were taken in range of 20-100 nM. Fluorescence was recorded at interval of 1 min for 10 min using Ex/Em 328/420. Michaelis-Menten curve was plotted between reaction velocity and substrate concentration to get V_{max} and K_m of the reaction. Lineweaver-Burk plot (double reciprocal plot) was used to find out the nature of inhibition. Three different concentrations of inhibitors **52** and **46** (0, 60 nM and 100nM) were taken with five different concentration (20-100nM) of substrate (Table S1, figure S3 and S4). The results were used to draw secondary plot for the determination of Ki of the compounds **52** and **46**(361). Enzyme kinetic assay was performed in triplicate.

4.1.6.5 *In-vitro* blood-brain barrier permeation assay

Permeability of the selected compounds across BBB was determined by parallel artificial membrane permeation assay (PAMPA-BBB)**(362)**. Porcine brain lipid (PBL) was purchased from Avanti polar lipids, Alabaster and dodecane was acquired from Avra Synthesis, Hyderabad. Acceptor microplates with PVDF membrane, pore size 0.45 µm and donor microplates were procured from Merck Millipore. The acceptor plate was coated with 4 µL of 20 mg/ml PBL in dodecane and filled with 200 µL of buffer (pH 7.4). Compounds **52** and **46**, 5 mg each, were dissolved in 1 ml of DMSO and 10 µL of the solution of both compounds were taken and were further diluted to 200 fold by buffer of pH 7.5 to get a final concentration of 25 µg/ml. 200 µL of the 25 µg/ml

compounds were added to the donor well plate. The acceptor plate was kept carefully over the donor plate like a sandwich and incubated for 18 h. The concentration of drug in the acceptor, donor and reference wells were determined by UV spectroscopy (HTX multi-mode reader, BioTek, USA) using microplate reader. Each of the sample was scanned to at least five different wavelengths and in three independent runs. PAMPA model was validated by using 9 (Verapamil, Diazepam, Progesterone, Atenolol, Dopamine, Lomefloxacin, Alprazolam, Chlorpromazine and Oxazepam) commercial drugs with known BBB permeability.

4.1.6.6 MC65 neuroprotection assay

MC65 cell was obtained as a kind gift from Dr. George M. Martin of University of Washington(363, 364). The MTT assay kit was purchased from Himedia. MC65 cells were grown in MEM, washed with PBS and were resuspended in MEM. Further, 5×10^4cells/well were plated in the 96 well plates. The cells were incubated with +TC and –TC in CO_2 incubator (Heal force, Germany). In the test group (70-1µM) TC was absent (TC-). Test compound **46** was added and cells were incubated at 37 ºC for 12 h. At the end of 12 h, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added (20 µL, 5 mg/mL) and the cells were incubated for another 4 h. Medium was removed and the formazan crystals produced were dissolved in 150 µL of dissolving solvent (DMSO:H_2O, 1:1). The absorbance was immediately recorded at 570 nm and response was expressed in percentage cell viability relative to +TC as a control. The assay was performed in triplicate and in three independent runs.

4.1.6.7 $A\beta_{1-42}$ inhibition assay

ROS was found to be produced by Aβ-redox active metal complex by Fenton-type reaction, which induced oxidative stress leading to death of neurons. The Aβ oligomers and aggregates formed with or without AChE and metal ion induce multifaceted toxicity

leading to neuronal cell death(365). Hence, a multifaceted approach is needed to overcome the toxicity produced by Aβ aggregates. ThT assay was performed to determine inhibition potential of compounds **52** and **46** against AChE induced $A\beta_{1-42}$ aggregation(365, 366). $A\beta_{1-42}$, purchased from Sigma, was dissolved in 10 mM phosphate buffer (PBS) of pH 7.5 to get a stock concentration of 200 mM. The test compounds were dissolved in the DMSO and further diluted with the PBS. Different proportions of the $A\beta_{1-42}$: Inhibitor (1:0.5, 1:1, 1:2) were used in ThT assay. The final concentration of $A\beta_{1-42}$, compounds **52**, **46** and AChE was 10 μM (2μL), 0.5, 10, 20 μM (2μL) and 230 μM (16 μL) respectively. The mixtures were incubated at room temperature for 48h in dark. The fluorescence intensities of the incubated mixtures were measured by adding 178 μL of 20 μM ThT at excitation and emission wavelengths of 485 and 528 nm at the end of experiment. The ThT assay was performed in triplicate and in three independent runs.

4.1.6.8 Antioxidant activity (DPPH assay)

2,2-Diphenyl-1-picrylhydrazyl, a stable free radical with purple color, pairs with antioxidants and forms yellow colored diphenylpicrylhydrazine. The assay measures free radical scavenging capacity of compound as a result of reduction of the DPPH by antioxidant. The assay was performed by previously mentioned protocol(367-369). Briefly, 10 and 20 μM (10 μL) of the synthesized compounds (Tris-HCl buffer, pH 7.4) were mixed with 20μL, 10mM (stock in methanol) of DPPH (Hi-Media) in 96 well plate. The volume of solution was adjusted to 200 μL using methanol. The 96 well plate was incubated at 37ºC for 25 min. on shaking water bath with moderate shaking. The absorbance of reaction mixture was measured at 520 nm wavelength. The reducing percentage (RP) of the DPPH was determined by the equation $RP = 100\ [(A_0 - Ac)/A_0]$, Where A_0 was the control or untreated DPPH absorbance and Ac was the test treated

DPPH absorbance. Ascorbic acid was used as the standard for the DPPH assay. The assay was performed in triplicate(370).

4.1.6.9 *In-vitro* metal chelation assay

Compound **46** (1.11 mg, 600 µM) was dissolved in 3 ml of deionized water and pH was monitored using p^H meter. Solution obtained was scanned under UV-visible spectroscopy in the range of 200-760 nm. $CuCl_2$ (600 µM) was dissolved in 3 ml of water to get a colorless solution. The two solutions, $CuCl_2$ and compound, were mixed, pH was monitored and scanned. Finally, the pH was raised to 7.4 by the addition of diisopropylethylamine (DIPEA) (100 µL DIPEA+ 900 µL DI water) and the mixture was incubated for 8 h. The graph were plotted on excel comparing shift in wavelength before and after complexation. Metal chelation assay was performed in triplicate.

4.1.6.10 Scopolamine induced amnesia model

4.1.6.10.1 Animals

Adult male Swiss Albino mice weighing 20-25 g were used in the study. The animals were kept in polyacrylic cages (22.5×37.5 cm) at room temperature (24-27 ºC) with 12 h dark and light cycle. The food was withheld 1h before the behavioral study. The procedure and number of animals required for the study were approved by the Institutional animal ethical committee (Protocol No. Dean/13-14/CAEC/342).

4.1.6.10.2 Materials

Scopolamine hydrobromide and donepezil were purchased from Sigma-Aldrich, the study was performed on Passive avoidance test apparatus (Make- Orchid scientific India). Tetramethylrhodamine (TMRM) obtained from Thermofisher.

4.1.6.10.3 Administration of scopolamine

Scopolamine hydrobromide (3mg/kg) was dissolved in distilled water and administered i.p on the seventh day, 1 h after test or donepezil administration. The behavioral study was performed 5 min after scopolamine injection(371).

4.1.6.10.4 Experimental protocol and drug administration

Animals were divided into ten groups containing 6 animals each. Donepezil and test compounds **52** and **46** were freshly dissolved in distilled water before dosing. Mice were divided into ten experimental groups of six animals each: (i) vehicle (1 ml) (ii) scopolamine (3mg/kg), (iii) scopolamine plus donepezil (1mg/kg), (iv) scopolamine plus compound **52** (5 mg/kg), (v) scopolamine plus compound **52** (10 mg/kg), (vi) scopolamine plus compound **52** (20 mg/kg), (vii) scopolamine plus compound **46** (5 mg/kg), (viii) scopolamine plus compound **46** (10 mg/kg), (ix) scopolamine plus compound **46** (20 mg/kg), (x) control. Dose of the compounds was fixed on the basis of their LD_{50}. The route of drug administration was intraperitoneal injection (i.p) for all the groups. Donepezil and compounds **52** and **46** 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 different set of animals were used for Y-Maze test and passive avoidance experiment.

4.1.6.10.5 LD_{50} determination

OECD guidelines for testing of chemicals was used to determine LD_{50} of compounds **52** and **46** in 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. (Table S3-S8). LD_{50} determination of compounds **52** and **46**.

4.1.6.11 Evaluation of memory function: Y-Maze Test

The test was performed to evaluate immediate working memory. The experimental design and doses used in study are already mentioned in experimental protocol and drug administration section. The compounds **52** and **46** were evaluated at 5, 10 and 20 mg/kg doses. The test was carried out on seventh days (last day) of the treatment. Training session of 15 min. was carried out after dosing in which animals were placed in Y-maze with novel arm closed. After four hour of training session main study was performed after 5 min. of scopolamine hydrobromide i.p. injection. In this session, animal was placed at the center of arm to explore all the three arms. The experiment was performed for 15 min. and entry of mice in each of the arms was recorded with in camera and repeated arm entry was considered as the sign of memory impairment. Further, consecutive arm choices (ABC, BCA, CAB not BAB) and novel arm entry were considered as memory improvement. The memory improvement score was calculated using the equation: % alternation = (number of alternations/ (total arm entries) – 2) X 100.

4.1.6.12 Passive avoidance test

The test is used to evaluate learning and memory in rodents with CNS disorders. The scopolamine was found to impair memory retention, when given to rodents before training in dark avoidance test. There were 10 groups of adult male Albino mice (35-40 gm) containing 6 animals in each group. Once a day, 5, 10 and 20 mg/kg i.p. dosages of compounds **52** and **46** were used for seven days of treatment. The animals were trained after five minutes of i.p. administration of scopolamine by placing them in the bright chamber of two chamber apparatus for training on seventh day. Acquisition period of 30 s was used for each animal and then the door of the dark chamber was opened and closed after entry of animal in the dark chamber. Animals were subjected to low

intensity foot shock of 0.5 mA for 10 s in dark compartment. Acquisition trial was followed by retention trials having a time interval of 24 h. Movement of animals from one chamber to other was recorded as transfer latency time (TLT) in seconds. In retention trials foot shock was not delivered to prevent reacquisition. Acquisition and retention trial was performed for 300 s(372, 373).

4.1.6.13 Mitochondrial membrane potential

4.1.6.13.1 Mitochondrial isolation

Brain isolated from the animals used in Y-Maze and Passive avoidance tests was used to obtain mitochondria. Mitochondria from the whole brain excluding cerebellum was isolated with a method previously described after suitable modification(374). Homogenized brain of test, vehicle and control groups in the isolation buffer was centrifuged at 1300g at 4ºC for 5 min. Isolation buffer was prepared by taking 215 mM mannitol, 75 mM sucrose, 0.1% w/v bovine serum albumin, 20 mM HEPES buffer and 1 mM of EGTA in 100 ml of distilled water and pH was adjusted to 7.2 with KOH. The supernatant was collected and pellet was re-suspended in isolation buffer and centrifuged as mentioned earlier. Two supernatants were collected, mixed and centrifuged 14,000g for 10 min.at 4 ºC to get a mitochondrial pellet. Pellet was washed with isolation buffer without EGTA and centrifuged at 14,000g for 10 min. to remove EGTA. Mitochondrial protein was estimated colorimetrically(375).

4.1.6.13.2 Evaluation of mitochondrial membrane potential in brain region

Tetramethylrhodamine (TMRM) was used to determine the mitochondrial membrane potential. The fluorescence produced by Rhodamine dye treated healthy mitochondrial protein was measured at an excitation λ 485±20 nm and emission λ of 528±20 nm(376). The data was expressed as fluorescence intensity /mg of protein. TMRM assay was performed in triplicate.

4.1.6.14 Neurochemical analysis

Animals were sacrificed and whole brain was isolated after completion of behavioral study. The brain was homogenized in 10 mM phosphate buffer (pH 7.4) and centrifuged for 15 min at 15000 rpm and 4 °C. The supernatants were used for the further determination of different biochemical parameters.

AChE, and cholinergic biomarker levels, were estimated in the brain of an animal by Ellman's method(358, 377). 100 µL of the supernatant was incubated with 15 mM of freshly prepared ATCI (100µL) in presence of 2.7 ml of phosphate buffer for 5 min. The absorbance was recorded at 415 nM after addition of 100 µL of 1.5 mM DTNB.

The toxic insult *i.e.,* H_2O_2 produced in the body, breaks down by the catalase (CAT) enzyme into oxygen and water. CAT activity was determined by mixing 100 µL of supernatant with 150 µL of 0.01M phosphate buffer (pH 7). The reaction was started by 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 prevent the reaction. The reaction mixture was kept on boiling water bath for 15 min and green color appeared was analyzed spectrophotometrically at 570 nm wavelength. Neurochemical analysis assay was performed in triplicate.

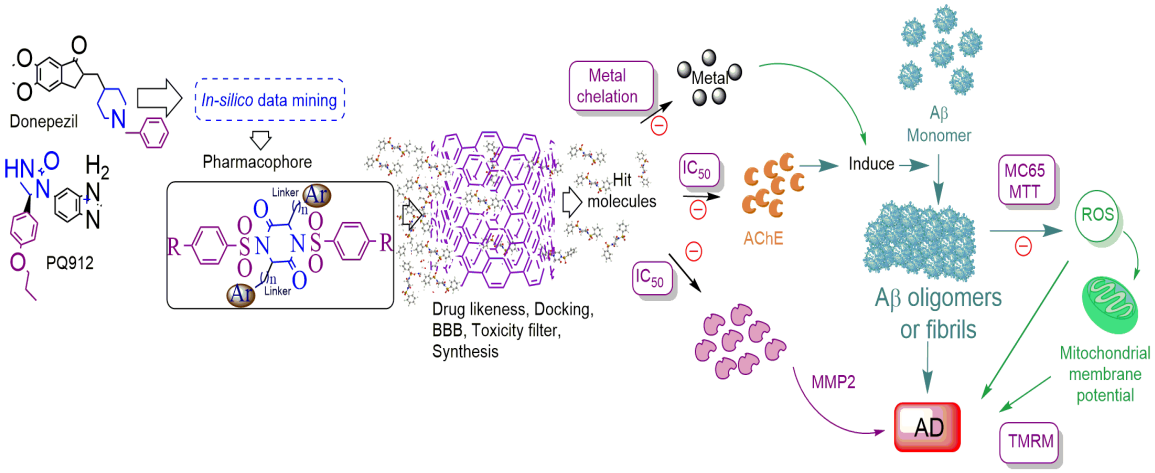
4.2 Result & discussion

4.2.1 Rational of drug design and discovery

Hybrid pharmacophore methodology and database mining approaches were used to design the novel compounds. FDA approved compounds *i.e.,* Donepezil and PQ912 (in phase II clinical trial drug for AD) were selected to identify essential pharmacophore features. The developed pharmacophores were subsequently used for database mining of "Zinc15" and "Asinex" databases. The similar pharmacophoric compounds obtained, were further subjected to various filters *i.e* drug-likeness property, *in-silico* BBB

permeability and toxicity. The selected compounds were docked to identify the functional groups and fragments to provide key interactions with the targets (AChE and MMP-2) (**Figure 4.1**).

Figure 4.1. Design of multitarget-directed potential drug candidates for AD.



4.2.2 Chemistry

Aromatic and aliphatic piperazine-2,5-diones were synthesized according to schemes I and II. Sulfonamide derivatives (**14-35**) were prepared by treating substituted aromatic sulfonyl chloride (**1-13**) with aliphatic amino acid (**11**) or aromatic amino acids (**12**, **13**) in presence of base. The substituted sulfonamides (**14-35**) were initially characterized by FTIR followed by ^{1}HNMR. FTIR showed characteristic secondary amine stretching band in the region of 3300-3200 cm^{-1} and presence of hump in the range of 2900-2800 cm^{-1}, carbonyl band appeared in between 1690-1650 cm^{-1} and sulfur dioxide band in between 1100-1070 cm^{-1}. Carboxy group was found above 9.0 ppm followed by aromatic, amino and methylene protons at 7-8, 4.5-5.5 and below 4.3 ppm respectively in ^{1}H-NMR. Piperazine-2,5-dione derivatives were synthesized by cyclization with different sulfonamides (**14-35**) using ethyleneglycol as a cyclizing agent. Compounds

60, 61 were synthesized by reacting substituted alphatic sulfonyl chlorides (**58, 59**) with glycine (**11**) in presence of sodiumbicarbonate and acetone-water mixture (Scheme II). The FTIR showed bands in between 3300-3200, 2800-2500, 1680-1630 and 1110-1030 cm^{-1} for a secondary amine, carboxy and sulfur dioxide respectively.

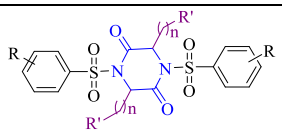
4.2.3 *In-vitro* biological evaluation

AChE, BuChE and MMP-2 inhibition assay: Considering the fact that AD is multifactorial in nature, we designed multifunctional drugs to address the basic factors responsible for the disease. The pharmacophore 1,4-bis(phenylsulfonyl)piperazine-2,5-dione (**36**) was the starting point for designing the potent compounds. The structural modifications were centered towards the introduction of various substituents on the peripheral phenyl and central piperazine rings. The first goal was to find out the significance of peripheral phenyl rings in the interaction with the enzymes. Therefore, compound **36** and its aliphatic analogs **62** and **63** were designed, synthesized and evaluated for AChE and MMP-2 inhibitions(355). In this assay, compound **36** having phenyl ring turned out to be potent in comparison to **62** and **63** (IC_{50}, AChE, **36** = 98.53±0.025 nM, IC_{50} **62** and **63** = >200.00 nM; IC_{50}, BuChE, **36** = >200.00 nM; IC_{50}, MMP-2, **36** = 82.17±0.024 nM, IC_{50} for **62** and **63** was>1000.00 nM, **Table 4-1**). This may be due to the aromatic-π interaction of molecule with the target. We further introduced various electron withdrawing groups (EWG) and electron donating groups (EDG) to the phenyl ring of lead molecule **36** to explore the role of substituents *p*-Cl, *m*-Cl, *p*-Br, *p*-F and *p*-CH_3 which led to generation of compounds **38**, **39**, **40**, **43** and **37**. Interestingly, compound **38** bearing chloride group at *para* position on the phenyl ring showed maximum inhibition of the enzymes over EDG analog **37** (IC_{50}, AChE = 61.62±0.094 nM,117.70±0.0930 nM for **38** and **37**, respectively; IC_{50}, BuChE = 180.21±0.230 nM, >200 nM for **38** and **37**, respectively; IC_{50}, MMP-2 =

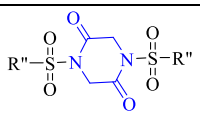
49.69±0.031nM, and 86.74±0.473nM, for **38** and **37** respectively). It was also observed that strong EWG (**43**) and larger size EWG (**40**) are not well tolerated on AChE binding site (IC_{50}, AChE = 78.32±0.042 nM, 81.84±0.522 nM for **43** and **40**, respectively) while no significant difference was observed in the inhibition of MMP-2 (IC_{50}, MMP-2 = 55.13±0.051nM, and 54.68±0.007 nM, for **43** and **40** respectively). This is also evident from structure of the binding interaction of the molecules, indicating that π-π and π-sulfur are crucial for activity. The role of hydrogen bonding in the interaction with targets was explored by introducing hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) groups at *para*-position of the lead molecule **36**. The amine containing compound **42** could inhibit MMP-2 at lower concentration compared to methoxy compound **41** (IC_{50}, MMP-2 = 53.14±0.014nM, and 75.25±0.022 nM, for **42** and **41** respectively). It is noteworthy that these analogs were found to be more active as compared to **36** (**Table 4.1**).

After optimizing the suitable substituents on the peripheral phenyl ring, we introduced aromatic feature into the central piperzaine ring. This modification is based on the observation of π-π interaction with amino acid residues Trp 86, 238 and Asp74 in AChE and Ala 80, 88 in MMP-2 with the aromatic ring. The addition of phenyl ring into the piperazine ring, generated compound **46** (IC_{50}, AChE = 28.65±0.029 nM; IC_{50}, BuChE = 160.58±0.082 nM; IC_{50}, MMP-2 = 19.57±0.005 nM) which was found to be active in comparison to the parent compound **36**. Interestingly, AChE inhibitory property of compound **46** was slightly less than donepezil (IC_{50} = 225.06±0.041 nM). Based on these results, we introduced phenyl ring into previously synthesized compounds bearing substitution on peripheral phenyl ring. In this series, total six compounds were synthesized. They were subjected to enzyme inhibition studies after characterization.

Table 4.1 Aromatic and aliphatic piperazine derivatives with inhibitory activities (IC_{50}) against AChE and MMP-2.



(36-57)



(62,63)

Comp	R	R’	n	AChE IC_{50} ±SE (nM)	BuChE IC_{50} ±SE (nM)	selectivity ratio[a]	MMP-2 IC_{50} ±SE (nM)
36	-H	-H	0	98.53±0.025	>200	0.5	82.17±0.024
37	-4CH_3	-H	0	117.70±0.030	>200	0.6	86.74±0.473
38	-4Cl	-H	0	61.62±0.094	180.21±0.230	0.3	49.69±0.031
39	-3Cl	-H	0	66.73±0.027	197.47±0.142	0.3	58.02±0.010
40	-4Br	-H	0	81.84±0.522	190.8±0.627	0.4	54.68±0.007
41	-4OCH_3	-H	0	91.48±0.014	>200	0.5	75.25±0.022
42	-4NH_2	-H	0	86.35±0.029	>200	0.4	53.14±0.014
43	-4F	-H	0	78.32±0.042	185.54±0.384	0.4	55.13±0.051
44	-4OH	- C_6H_5	0	84.25±0.018	>200	0.4	57.24±0.040
45	-4NH_2	-C_6H_5	0	91.33±0.017	>200	0.5	50.55±0.021
46	-4Cl	- C_6H_5	0	28.65±0.029	160.58±0.082	0.2	19.57±0.005
47	-4NO_2	-C_6H_5	0	60.76±0.058	175.81±0.172	0.3	33.47±0.027
48	-4CH_3	-C_6H_5	0	76.39±0.032	>200	0.4	62.87±0.074
49	-3CH_3	-C_6H_5	0	83.86±0.021	>200	0.4	73.82±0.128
50	-4Br	-C_6H_5	0	50.62±0.023	183.25±0.251	0.2	32.71±0.005
51	-3Br	-C_6H_5	0	76.64±0.070	192.74±0.299	0.4	54.74±0.085
52	-4Cl	-C_6H_5	1	32.45±0.044	157.95±0.264	0.2	36.83±0.015
53	-4OH	-C_6H_5	1	59.45±0.132	198.65±0.284	0.3	72.02±0.103
54	-3OH	-C_6H_5	1	69.80±0.020	>200	0.3	83.04±0.021
55	-4NO_2	-C_6H_5	1	57.35±0.024	162.57±0.451	0.4	53.82±0.042
56	-4Br	-C_6H_5	1	56.97±0.021	180.75±0.454	0.3	38.68±0.045
57	-3Br	-C_6H_5	1	76.75±0.041	190.62±0.571	0.4	58.08±0.024
62	-----	-C_4H_9 (**R”**)	--	>200.00	>200.00	--	>1000.00
63	-----	-C_3H_7 (**R”**)	--	>200.00	>200.00	--	>1000.00
NNGH	-----	-----		-----	-----	--	14.00±0.001
DNP	-----	-----	--	21.08± 0.036	-----	--	-----

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

As expected, the phenyl piperazine derivatives **48**, and **50** (IC_{50}, AChE = 76.39±0.032 nM, 50.62±0.023 nM for **48** and **50**, respectively; IC_{50}, MMP-2 = 62.87±0.074 nM, and 32.71±0.005nM, for **48** and **50**, respectively) inhibited both the enzymes with lower IC_{50} values as compared to piperazine analogs, **37** and **40**, respectively (IC_{50}, AChE = 117.70±0.030 nM, 81.84±0.522 nM for **37** and **40**; IC_{50}, MMP-2 = 86.74±0.473 nM, and 54.68±0.007 nM, for **37** and **40** respectively). It is important to point out that slight difference among the amine containing phenyl piperazine **45** and its parent compound **42** (IC_{50}, AChE = 91.33±0.017nM, 86.35±0.029 nM for **45** and **42**, respectively; IC_{50}, MMP-2 = 50.55±0.021nM, and 53.14±0.014 nM, for **45** and **42** respectively) was noticed which could be due to complex interaction of amine with the target. In this series, hydroxyl and nitro derivatives, **44** and **47**, were also tested and found to be potent inhibitor of enzymes (IC_{50}, AChE = 84.25±0.018 nM, 60.76±0.00 nM for **44** and **47**, respectively; IC_{50}, MMP-2 = 57.24±0.040 nM, and 33.47±0.027 nM, for **44** and **47**, respectively). However, the chloride bearing **46** remained most potent so far in this series. Finally, we introduced one carbon linker between phenyl and piperazine to the Tyr124, 341 in AChE and His120, Leu83, Ala84 in MMP-2. The six molecules synthesized in this category were less active against MMP-2 in comparison to the molecules without a linker. The chlorine containing compound **52** lost the activity by 10% compared to **46** (IC_{50}, AChE = 32.45±0.044 nM, 28.65±0.029 nM for **52** and **46** respectively). However, the bromo compounds, **56**, **57** and nitro compound **55** retained the activity against AChE (IC_{50}, 56.97±0.021 nM, 76.75±0.041 nM and 57.35±0.024 nM for **56**, **57** and **55**, respectively) while a small reduction in MMP-2 activity was observed (IC_{50}, 38.68±0.045 nM, 58.08±0.024 nM and 53.82±0.042 nM for **56**, **57** and **55**, respectively). The *para*-hydroxyl compound **53** gained the activity in comparison to

its analog **44** against AChE (IC_{50}, 59.45±0.132 nM, 84.25±0.018 nM for **53** and **44**, respectively).

The mechanism of AChE inhibition was studied in detail by exploring the enzyme kinetics parameters of compound **46**. V_{max} and V_{max}/K_m in the Michaelis-Menten graph was found to decrease by increasing the concentration of inhibitor, while K_m increased (**Figure 4.2**). The pattern of V_{max} and K_m along with Lineweaver-Burk plot suggested that compound **46** is acting on both free enzyme and enzyme-substrate complex (noncompetitive inhibition). In Lineweaver-Burk plot, the intersect at value of 1/[S] less than zero and 1/[V] greater than zero thus $\alpha > 1$ indicated that the inhibitor preferentially bound to the free enzyme rather than enzyme-substrate complex (**Figure 4.3**). The Dixon plot showed that compound **46** had Ki = 7 nM (**Figure 4.4**).

The mechanism of MMP-2 inhibition was established by kinetic study. Compound **46** inhibited enzyme by competitive mode of action as V_{max} of enzyme remained constant throughout the enzyme kinetics at different concentrations of inhibitor. The V_{max}/K_m decreased gradually on increasing the inhibitor concentration (**Figure 4.5**). The value of dissociation constant (K_i) of the enzyme reaction was determined by the graphical method(360) by plotting K_m*vs*[I] (I, inhibitor). The inhibitor was found to bind tightly with the enzyme having Ki value of 20 nM (**Table 4.2, Figure 4.6** and **4 7**).

4.1.6 Blood-brain barrier permeation assay (PAMPA)

Permeability of synthesized compounds for blood-brain barrier (BBB) is a prerequisite to target AD. Therefore, the compounds were screened by parallel artificial membrane permeation assay (PAMPA)(362). PAMPA model can successfully determine the passive BBB permeation with high accuracy. The *in-vitro* permeability of the synthesized compounds and nine commercially available drugs was performed through porcine brain lipid (PBL) dissolved in dodecane.

Figure 4.2 Michaelis-Menten plot at seven different concentrations of substrate (20-70 nM) on three different concentrations of compound **46**.

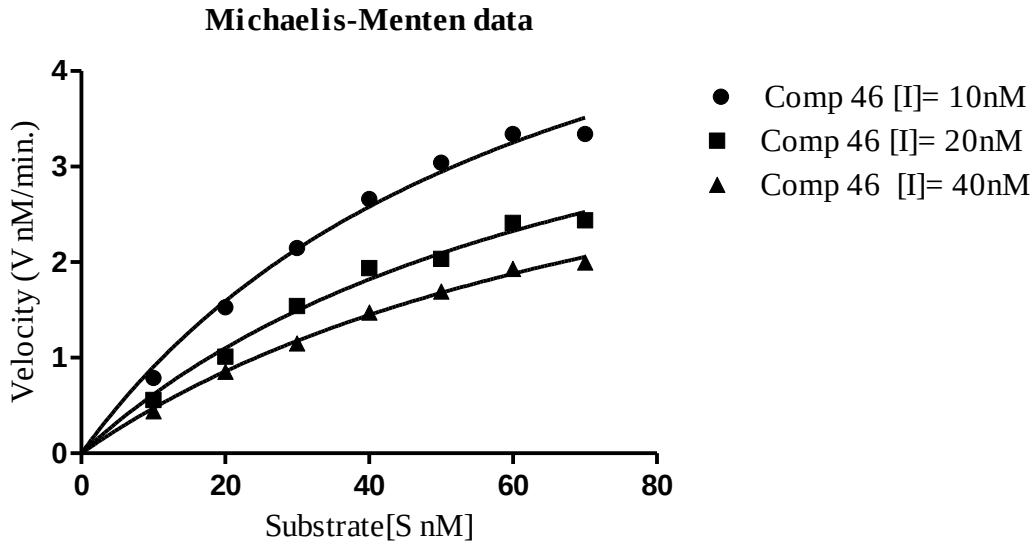


Figure 4.3 Lineweaver-Burk plot on three different concentrations of compound **46** for AChE: V_{max}, Km and V_{max}/K_m at 10 nM, 20 nM and 40 nM are found to be 6.787±0.7669 U/min, 5.239±0.7450 U/min, 4.665±0.4052 U/min and 65.26±12.80 nM, 75.19±17.63 nM, 88.95±12.04 nM and 0.1039, 0.0696, 0.00052 respectively. The K_m of the enzyme kinetics suggests that α >1 (αKi)**.**

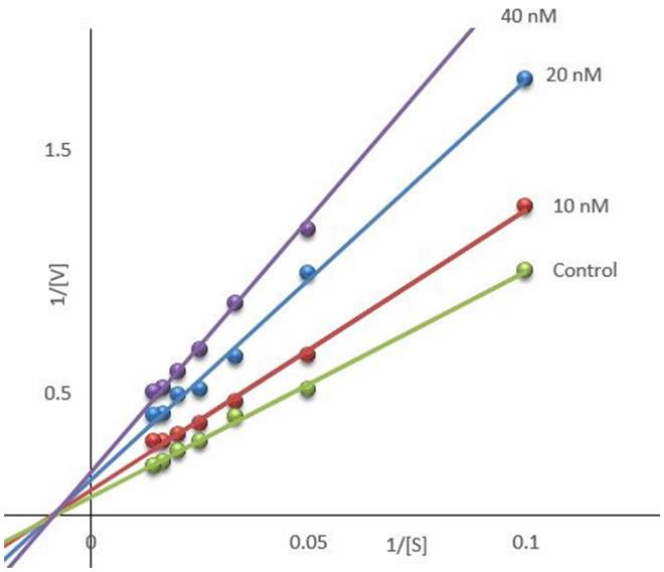


Figure 4.4 Dixon plot of compound **46** showing Ki value as negative intercept on X-axis of the Dixon plot for AChE.

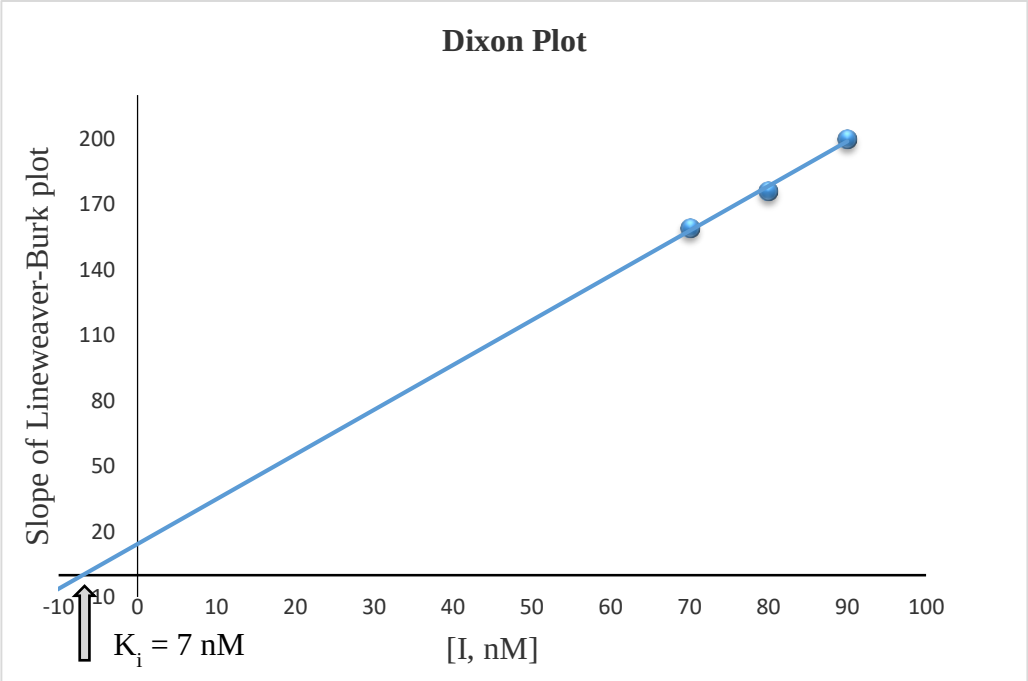


Figure 4.5 Lineweaver-Burk plot on three different concentrations of compound **46** for MMP-2 enzyme: Pattern of intersecting lines indicated competitive inhibition. V_{max} at various inhibitor concentration was found to be approximately constant (3.4 U/min). K_m and V_{max}/K_m at 0 nM, 60 nM and 100 nM of inhibitor was found to be 25.00± 7.021 nM, 36.00± 5.052 nM, 70.00± 9.037 nM and 0.136, 0.094, 0.049 respectively.

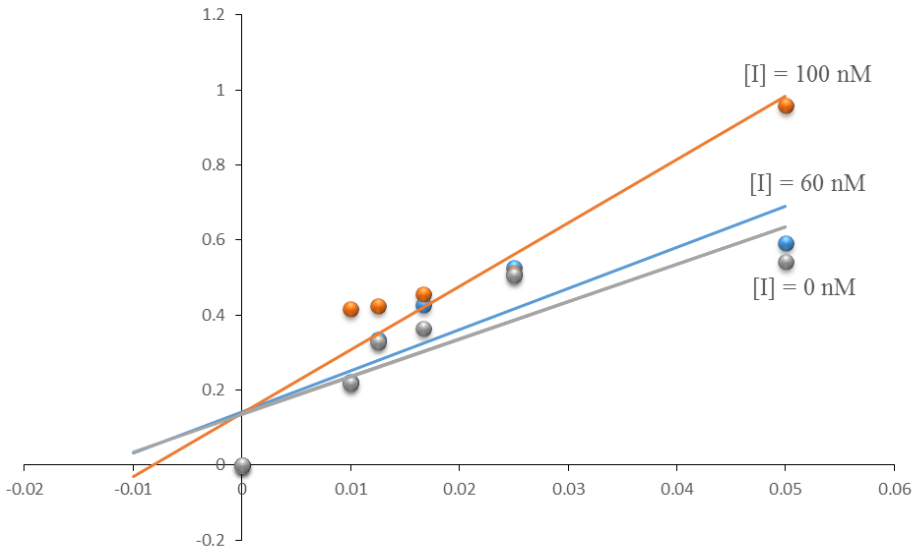


Table 4.2 Measurement of initial velocity of AChE as a function of substrate concentration at an inhibitor concentrations of 0, 60 nM and 100 nM.

Compound 52			Compound 46		
[I], nM	**Km**	**Vmax, nM/min**	**[I], nM**	**Km**	**Vmax, nM/min**
0	10	3.6	0	25	3.4
60	24	3.6	60	36	3.4
100	48	3.6	100	70	3.4

Figure 4.6 Determination of Ki against MMP-2 of compound **46** (Ki = 20nM).

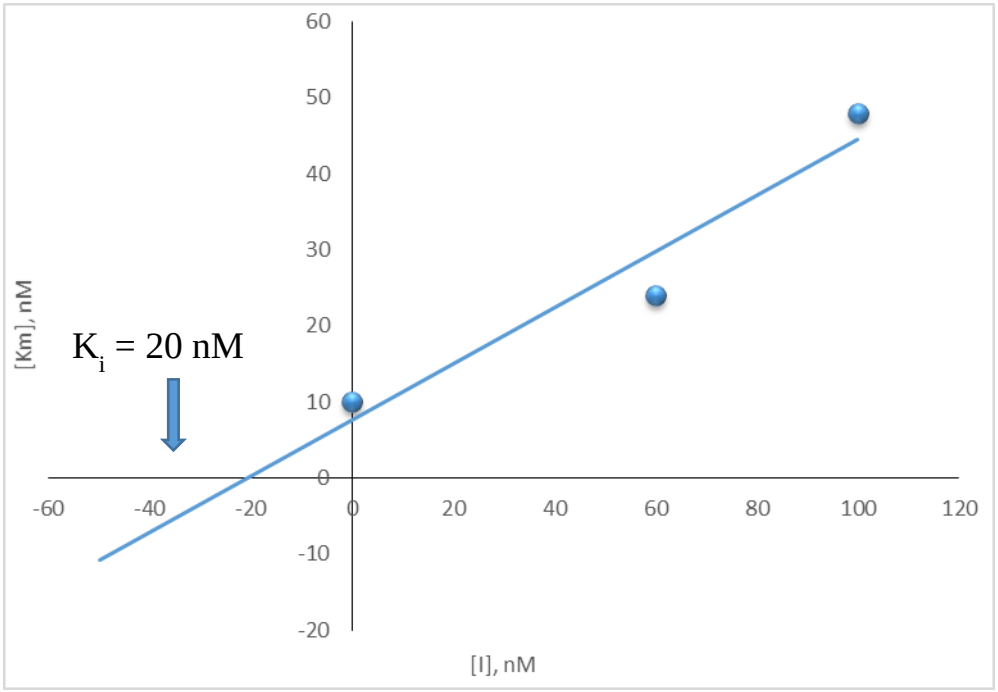
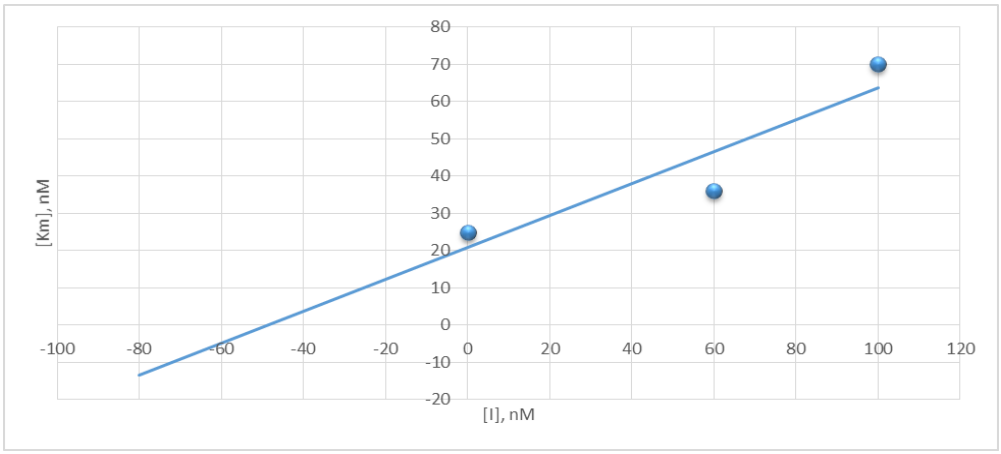


Figure 4.7 Secondary plot of Km as a function of inhibitor concentration [I] for a competitive inhibitor (compound **52**).



A linear correlation was established by using experimentally determined permeability (Pe) and reference (Pe). The relationship used to determine the cutoff for CNS permeability was Pe(exp) = 1.308Pe(literature)-0.8394, (R2 = 0.9317). Pe > 4.3×10-6 cm/s was for high CNS permeability (**Table 4.3**, **Figure 4.8**). Compounds **37**-**43** showed appreciable CNS permeability, but compounds **62** and **63** had very low CNS permeability (**Table 4.4**).

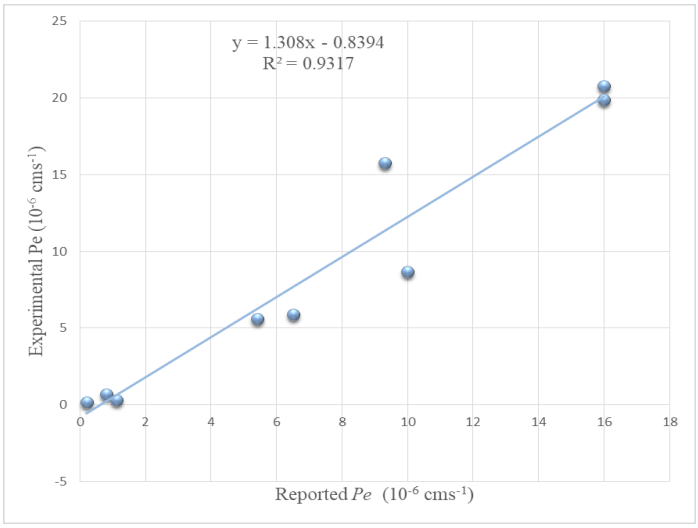
Table 4.3 Permeability (Pe×10-6 cm/s) of the 9 commercial drugs to validate the PAMPA-BBB model.

S.no.	Compounds	Reference† (Pe×10^{-6} cm/s)	Observed‡ (Pe×10^{-6} cm/s)
1	Verapamil	16	20.81±1.2
2	Diazepam	16	19.9±0.5
3	Progesterone	9.3	15.79±1.4
4	Atenolol	0.8	0.68±0.4
5	Dopamine	0.2	0.17±0.1
6	Lomefloxacin	1.1	0.31±0.3
7	Alprazolam	5.4	4.6±0.7
8	Chlorpromazine	6.5	5.9±0.6
9	Oxazepam	10	8.7±1.1

†Reference data taken from the Di *et al.*

‡Experimental result obtained from commercial drugs, data are the mean ± SD of three independent experiments.

Figure 4.8 Linear correlation between reported and observed Pe of the commercial drugs by PAMPA assay.



- Pe(exp) = 1.308Pe(literature)-0.8394 (R^2 = 0.9317).
- Pe (exp) (10^{-6} cms^{-1})> 4.3926(10^{-6} cms^{-1}) high BBB permeable.
- Pe (exp)(10^{-6} cms^{-1}) in between 4.3926-1.7766 (10^{-6} cms^{-1}) BBB permeability unpredictable.
- Pe (exp) < 1.7766 (10^{-6} cms^{-1}) low BBB permeable.

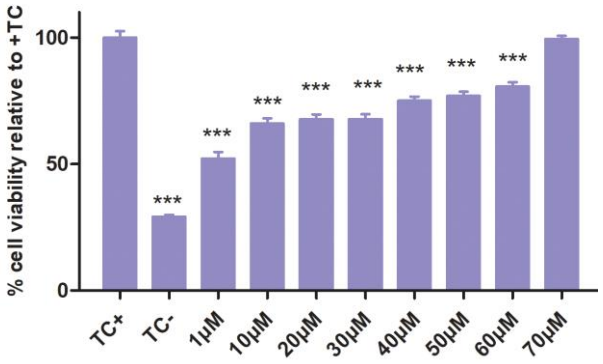
4.1.7 Neuroprotection studies

Human neuroblastoma MC 65 cell lines yield $A\beta_{1-40}$ by conditional expression of *C*-terminal APP protein. It mediates cell cytotoxicity by DNA fragmentation which is induced by ROS(378). The cell line suffers with oxidative stress and Aβ induced cellular toxicity in tetracycline removal condition (-TC). Therapeutic suitability and effectiveness of the molecules to prevent oxidative stress was determined by MTT assay. Most active compound **46** (70-1µM) was selected for neuroprotection and cytotoxicity studies. The neuroprotective ability of compound **46** was comparable to that of tetracycline (TC+). The percentage cell viability of compound **46** was found to be 99.41 in relation to +TC (**Figure 4.9**). MTT assay ascertained that molecule had the ability to compensate the oxidative stress generated in the absence of TC.

Table 4.4 Permeability, Pe (10-6 cm s1) determined by BBB-PAMPA study and DPPH based free radical scavenging activity of synthesized compounds.

Comp.	**Pe (10^{-6} cm s^{-1})[a]**	**Prediction[b]**	**RP of DPPH assay**	
			10µM	**20µM**
37	10.41±0.38	CNS+	34.8±0.07	46.7±0.23
52	12.33±0.13	CNS+	48.1±0.18	60.0±0.10
44	6.84±0.05	CNS+	52.9±0.16	65.2±0.19
38	6.43±0.26	CNS+	36.2±0.14	55.2±0.12
39	8.37±0.23	CNS+	35.7±0.49	55.2±0.15
40	8.87±0.17	CNS+	47.1±0.21	60.4±0.49
45	5.53±0.41	CNS+	56.2±0.14	66.7±0.21
41	6.46±0.02	CNS+	48.6±0.28	57.6±0.23
42	6.10±0.12	CNS+	55.7±0.21	62.9±0.28
46	7.38±0.34	CNS+	52.9±0.26	60.3±0.49
53	5.68±0.37	CNS+	58.1±0.14	68.6±0.14
54	6.36±0.16	CNS+	57.1±0.19	66.2±0.21
55	4.49±0.64	CNS+	42.9±0.21	54.8±0.07
47	4.63±0.03	CNS+	42.4±0.35	54.3±0.18
56	7.29±0.21	CNS+	47.6±0.18	64.8±0.14
57	8.06±0.06	CNS+	46.7±0.10	64.3±0.29
48	11.33±0.12	CNS+	38.1±0.17	56.2±0.09
49	11.85±0.15	CNS+	38.6±0.16	55.2±0.05
50	8.31±0.38	CNS+	45.7±0.15	63.8±0.15
51	9.45±0.52	CNS+	46.2±0.24	63.3±0.07
36	10.46±0.04	CNS+	39.5±0.29	52.4±0.24
43	10.34±0.25	CNS+	37.1±0.28	50.5±0.17
62	1.06±0.02	CNS-	65.7±0.25	56.7±0.28
63	0.89±0.05	CNS-	31.4±0.24	59.2±0.21
Ascorbic acid			37.1±0.31	48.8±0.13
Donepezil			60.1±0.24	74.6±0.05

Figure 4.9 MC65 neuroprotection with compound **46**: MC65 cells treated with Compound **46** at selected concentrations in absence of TC. TC+ was taken as control (One-way ANOVA followed by Newman-Keuls multiple comparison test compare all pair of column ** $p < 0.0001$).



4.1.8 AChE-induced $A\beta_{1-42}$ aggregation assay

Metal ions (Fe, Cu, Zn) and AChE are the two major factors responsible for Aβ aggregation. Peripheral anionic binding site (PAS) provides the seeding site for the low molecular weight oligomers(379). Aβ and AChE form complex which promotes the neurite network dystrophia and apoptosis. Furthermore, Aβ-AChE complex increases intracellular Ca^{2+} resulting in an imbalance of mitochondrial membrane potential(380).

A fixed amount of Aβ (10 μM) and variable concentrations of donepezil, compounds **52** and **46** (0.5, 10 and 20 μM) were used in the Thioflavin T (ThT) assay. Aβ and AChE with Aβ were taken as negative control. Donepezil, compounds **52** and **46** did not show significant results in the assay at 0.5 μM concentration. Donepezil showed over 60% of Aβ aggregation at 10 μM concentration while, compounds **52** and **46** showed less than 60% aggregation at same concentration. Further, Aβ aggregation was approximately 50% at 20 μM for donepezil but was less than 50% and 40% in case of compounds **52**

and **46**, respectively (**Figure 4.10**). ThT assay clearly indicated that compounds **52** and **46** were more potent than donepezil at 10 and 20 µM concentrations.

It was observed, in the kinetic study, that designed compounds showed non-competitive inhibition and thus were more efficient in inhibition of the seeding of Aβ oligomers. The compounds also showed metal chelating ability and might inhibit the metal induced Aβ aggregation. The potent nature of compounds **52** and **46**, compared to donepezil in Aβ aggregation, might be due to their dual mechanism of action. The hexapeptide sequence responsible for nucleation of Aβ was found to be KLVFFA(381). Compounds **52** and **46** depicted interaction (A chain) with Aβ protein by amino acids valine 24 (V24), phenylalanine 19, 20 (F 19, 20), alanine 21 (A21) and lysine 16(K16), leucine 17 (L 17), valine 18 (V18), phenylalanine 19, 20 (F 19, 20) respectively (**Figure 4.11**).

Figure 4.10 Effect of DNP, compounds **52** and **46** on AChE induced Aβ aggregation: Donepezil (DNP) compound **52** and **46** showed a concentration-dependent decrease in the Aβ aggregation; AChE-induced the Aβ aggregation, and it is decreased by the DNP, compound **52** and **46** (One-way ANOVA followed by one-way analysis of variance*** $p < 0.0001$), error bars represent the standard deviation (SD) of the normalized fluorescence intensity (NFI).

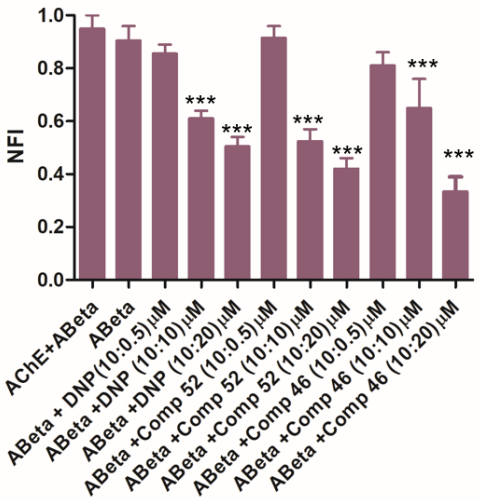
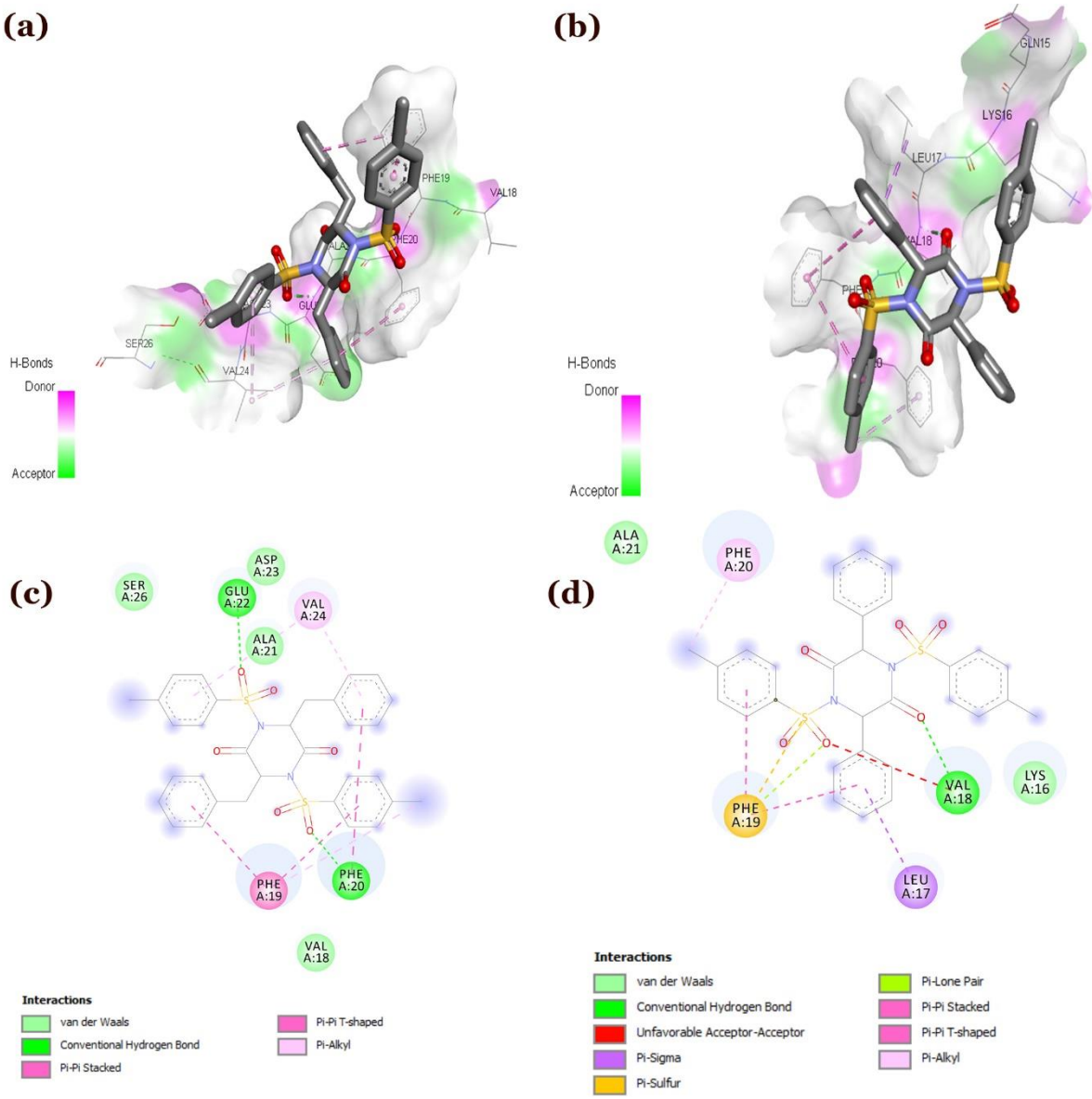


Figure 4.11 Docking poses of compound **52** (a, c) and **46** (b,d) against Aβ (PDB: 2LMN).



4.1.9 Antioxidant activity evaluation

DPPH assay is a reliable method to determine active oxygen scavenging property of compounds. The synthesized piperazine-2,5-diones were evaluated for their antioxidant potential by DPPH assay. Compounds **44**, **45**, **42**, **53**, and **54** displayed good antioxidant property (**Table 4.4**). The compounds containing -OH or -NH_2 type of donor groups are potent oxygen scavenger over other molecules in this series(370). Compounds **44, 53,**

54 containing a hydroxyl group and compound **45** having an amino group also participated in active oxygen scavenging. Rest of the molecules showed satisfactory DPPH reduction potential.

4.1.10 *In-vitro* metal chelation assay

Biometals (mainly Cu) are found to be involved in Aβ aggregation. The toxic oligomeric state of Aβ is stabilized by Aβ-metal complex(382). Cu^{2+} and Fe^{2+} bound Aβ produces ROS which induces oxidative stress followed by the neuronal death(383). The synthesized compounds were investigated for their metal chelation property by using $CuCl_2$. Compound **46** showed copper complexing property as observed in UV and FTIR spectra. The UV spectra of copper complex had a remarkable shift from the parent compound (**Figure 4.12**). FTIR spectra displayed change in sulfur dioxide peak indicating that it was involved in metal chelation (**Figure 4.13**) (384, 385).

Figure 4.12 The metal chelating property of compound **46**: The shift in UV spectra indicated the metal chelating property.

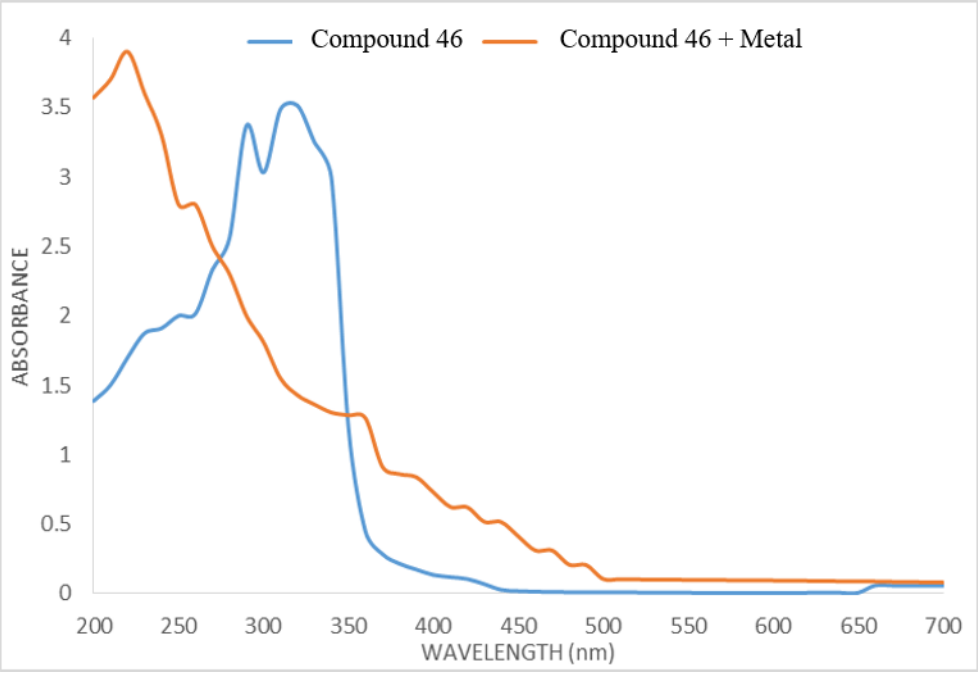
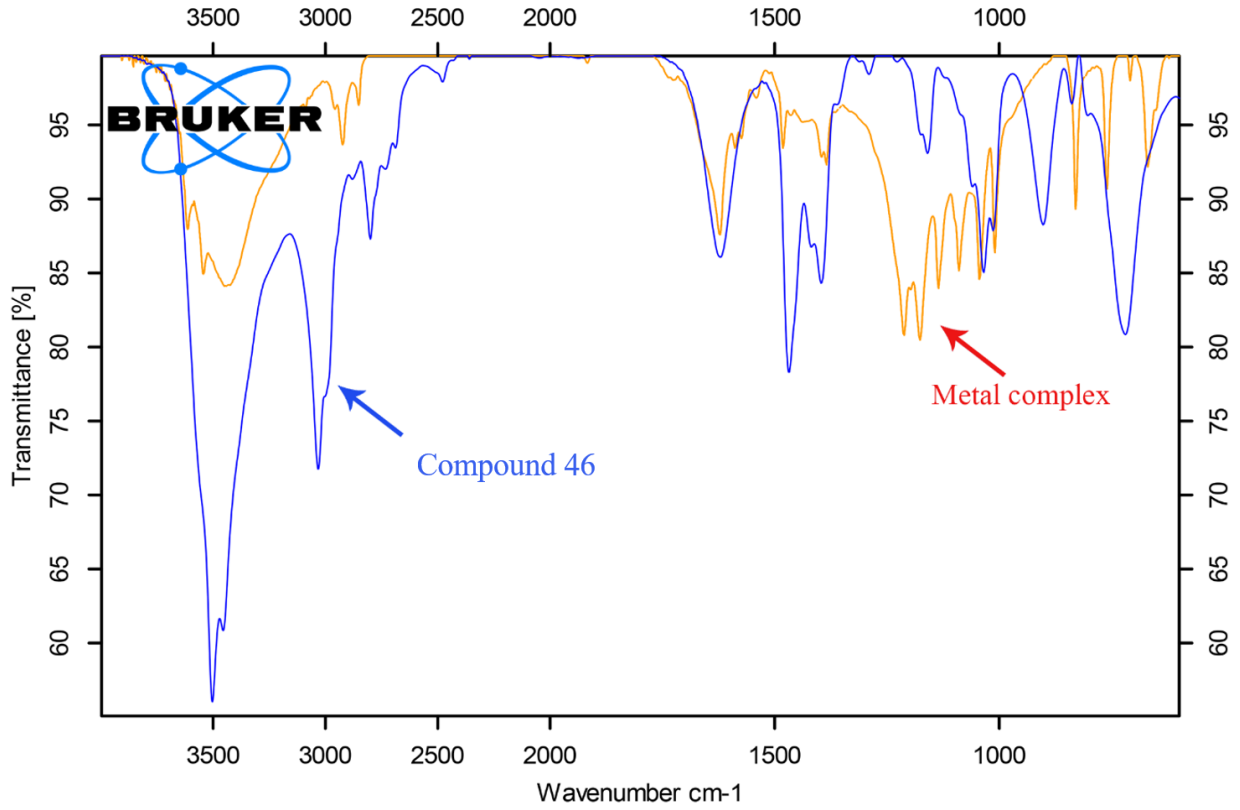
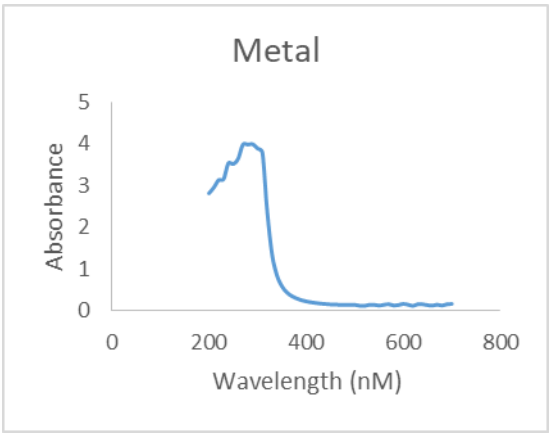


Figure 4.13 (**A**) FTIR spectra of the metal complex and compound **46** showing the shift in the bands. (**B**) UV-visible spectra of metal alone (**C**) UV spectra of buffer.

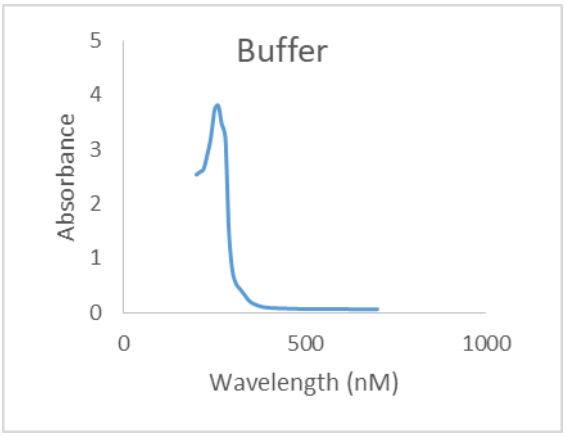
A



B



C



4.1.11 Behavioral Studies

Compounds **52** and **46** showed promising *in-vitro* activity and were further evaluated in AD animal models. The *in-vivo* scopolamine model was used to evaluate the anti-cholinesterase and dementia conditions in animals(372). Antiamnesic effect of compounds **52** and **46** was assessed by Y-Maze and passive avoidance tests. The dose of the compounds was fixed at half of the lethal dose (LD_{50}) data (**Table 4.5** to **4.10**).

Table 4.5 LD_{50} determination protocol for compound **52**.

Test substance	
1. Physical nature	Solid
2. Code	Compound **52**
Vehicle	water
Test animals	Rat
1. Sex	Female
2. Number	3
Test conditions	
1. Dose	300 mg/kg
2. Rationale for the selection of the starting dose	1 death at 50mg/kg
	0.6 ml
3. Dosing volumes	12 am 23/03/2017
4. Time & date of dosing	

Table 4.6 Effect of compound **52** on body wt. of animals at dose of 300 mg/kg.

Group	**Body wt. (gm) on 23/03/2017 at 12 pm**	**Body wt. (gm) on 24/03/2017 at 12 pm**	**Body wt. (gm) on 25/03/2017 at 12 pm**
1	200	198	198
2	240	239	238
3	217	215	213

Discussion and interpretation of results Animals are dosed as per OECD guideline 423 at 50 mg/kg and 300 mg/kg dose. All animals died at 300mg/kg dose within 72 hrs.

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

Table 4.7 Onset of toxicity with compound **52** in 72h.

Group	Body wt. Changes (gm)			Onset of toxicity	Reversibility	Date & time of death
	23/3/17	24/3/17	25/3/17			
1	00	02	0	23/03/17, 9 pm	No	25/03/17, 6PM
2	00	01	01	23/03/17, 9 pm	No	25/03/17, 6PM
3	00	02	02	23/03/17, 9 pm	No	25/03/17, 6PM

Table 4.8 LD_{50} determination protocol for compound **46**.

Test substance	
3. Physical nature	Solid
4. Code	Compound 46
Vehicle	water
Test animals	Rat
3. Sex	Female
4. Number	3
Test conditions	
5. Dose	300 mg/kg
6. Rationale for the selection of the starting dose	1 death at 50mg/kg
7. Dosing volumes	0.6 ml
8. Time & date of dosing	12 am 23/03/2017

Table 4.9 Effect of compound **46** on body wt. of animals at 300 mg/kg.

Group	Body wt. (gm) on 23/03/2017 at 12 pm	Body wt. (gm) on 24/03/2017 at 12 pm	Body wt. (gm) on 25/03/2017 at 12 pm
1	200	200	198
2	195	193	193
3	200	198	198

Table 4.10 Onset of toxicity with compound **46** in 72h.

Group	Body wt. Changes (gm)			Onset of toxicity	Reversibility	Date & time of death
	23/3/17	24/3/17	25/3/17			
1	00	00	02	24/03/17, 10 am	No	25/03/17, 9PM
2	00	02	00	24/03/17, 10 am	No	25/03/17, 9PM
3	00	02	00	24/03/17, 10 am	No	25/03/17, 9PM

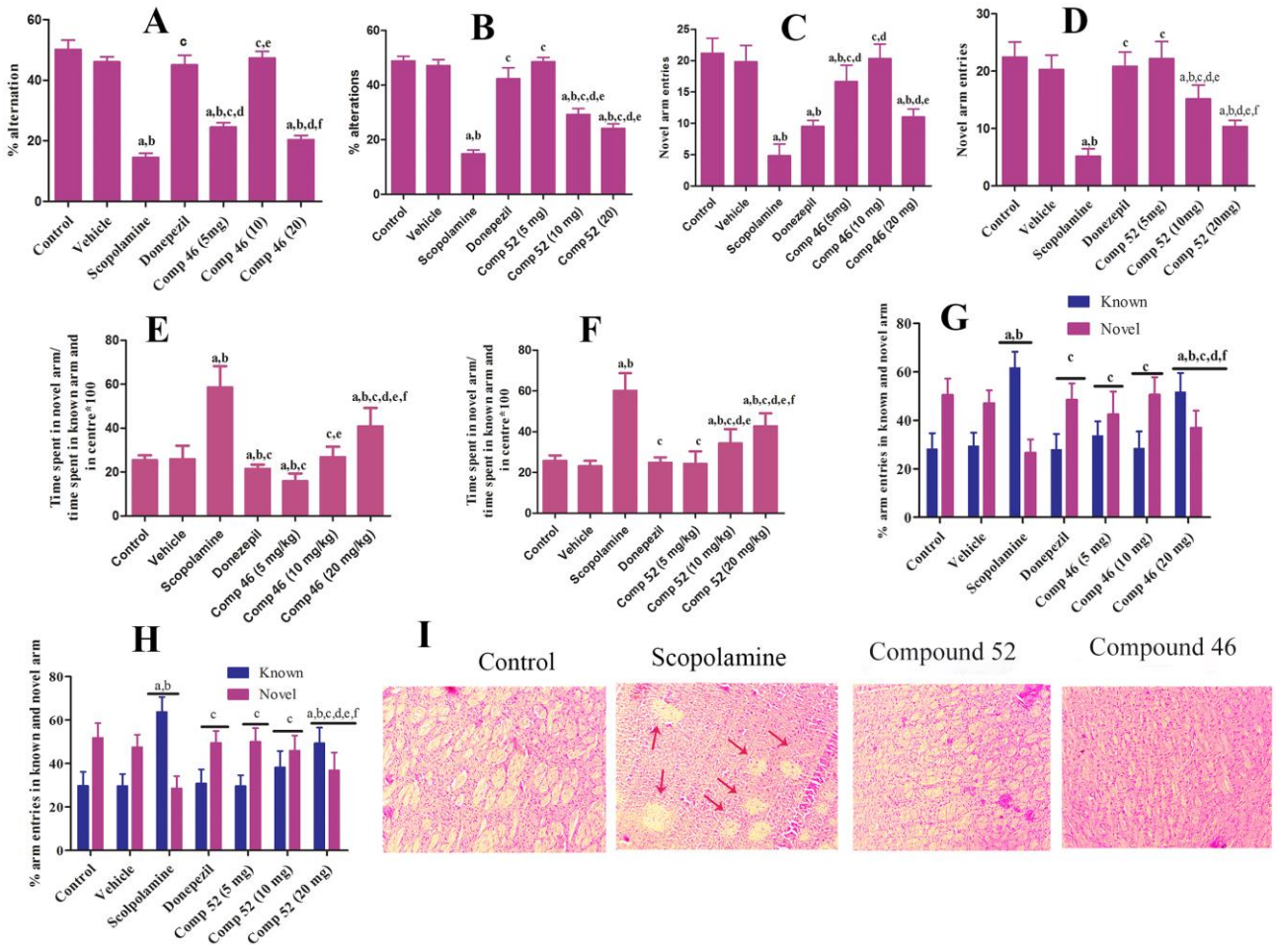
Discussion and interpretation of results Animals are dosed as per OECD guideline 423 at 50 mg/kg and 300 mg/kg doses. All animals died at 300mg/kg dose within 72 hrs.

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

4.1.12. Y-Maze Test

The working memory is assessed by spontaneous alteration score(386). Spontaneous alternation score (% alternation) was significantly decreased in scopolamine treated group as compared to control, vehicle and donepezil groups. Animals treated with compound **52** showed an increase in spontaneous alternation score at dose of 10 mg/kg, while compound **46** treated animals showed approximately same alternation score at 5mg/kg dose (**Figure 4.14 A, B**). Neophobia and anxiety behaviors of animals were determined by monitoring their novel arm entries. Compound **52** decreased neophobia and anxiety significantly at 10 and 5 mg/kg dosages as compared to scopolamine. Neophobia and anxiety behavior was also decreased by the compound **46** at 5 mg/kg dose, but its action decreased upon increase in the dose (**Figure 4.14 C, D**). The anxiolytic pattern of compounds **52** and **46** were further evaluated by observing the time spent by animals in novel arm, known arm and center of Y maze. The animals treated with 20 mg/kg of compound **52** spent significantly more time in the novel arm as compared to the control and vehicle (**Figure 4.14 E**). Moreover, at the same dose, compound **46** showed comparatively same anxiolytic activity (**Figure 4.14 F**). Further studies indicated that scopolamine treated animals spent most of their time in known arm as compared to novel arm indicating that scopolamine did not affect the locomotive behavior of animals. Compounds **52** and **46** improved working memory, decreased neophobia and anxiety but did not alter the locomotive behavior of animals (**Figure 4.14 G, H**).

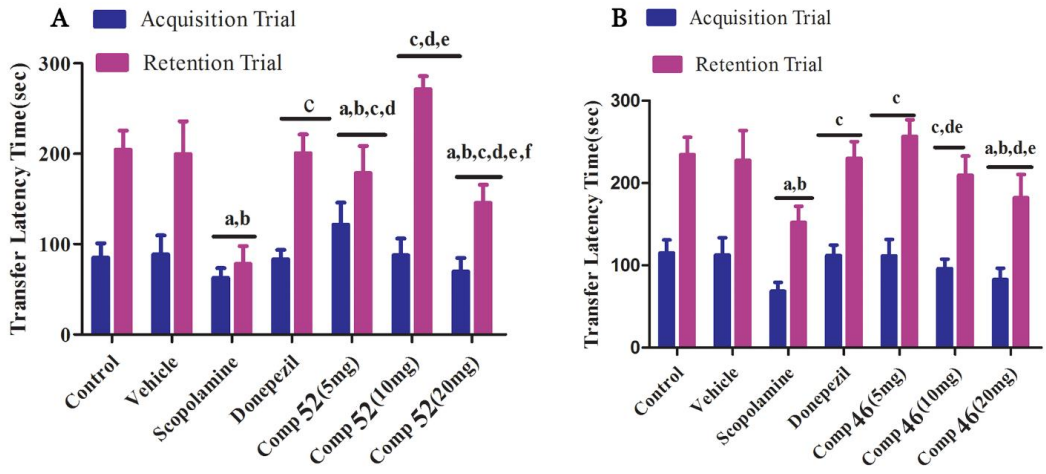
Figure 4.14 Effect of compounds **52** and **46** (5, 10, 20 mg/kg) and scopolamine on(A, B)Spontaneous alternation score(% alternation); (C, D) Novel arm entries of the animals; (E, F) Time spent in novel arm/time spent in known arm and in center*100; (G, H) % arm entries in known and novel arm in Y maze paradigm; (I) Histopathology of the brain. Bars show 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; $^{e}p < 0.05$ compared to compounds **52** or **46** at dose of 5 mg/kg; $^{f}p < 0.05$ compared to compound **52** or **46** at dose of 10 mg/kg.



4.1.13 Passive avoidance test

Learning response is mainly governed by hippocampus and central cholinergic neurons are involved in passive avoidance learning(387). The transfer latency time (TLT) was significantly increased in retention trial of control, vehicle and compounds **52** and **46** at doses of 10 and 5 mg/kg. Intraperitoneal (i.p.) injection of scopolamine caused cognitive impairment hence there was no significant change in the TLT of retention trial as compared to acquisition trial. Treatment with compounds **52** and **46** showed significant dose related TLT response (**Figure 4.15 A**, **B**). Compound **52** showed an increase in TLT, that was comparable to control, vehicle and donepezil at 10 mg/kg. Further, nearly similar response was observed at half (5 mg/kg) dose of compound **46**. The increase in the TLT at lower dose indicated that compound **46** was more potent than compound **52**.

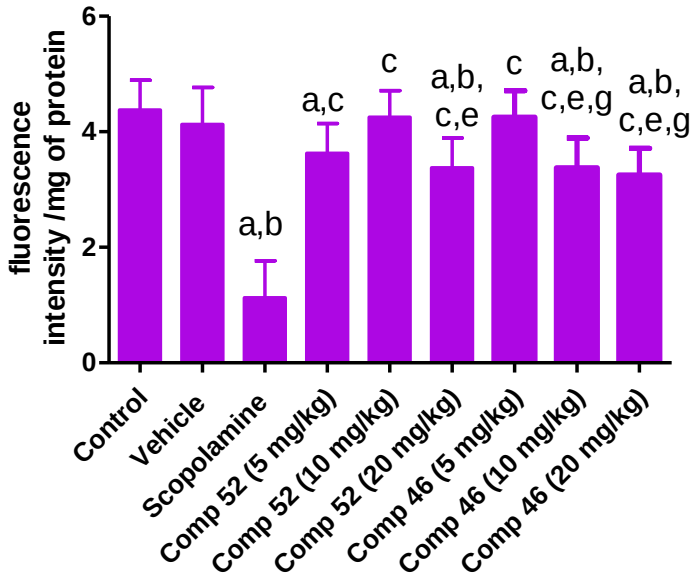
Figure 4.15 Effect of scopolamine induced alteration in the passive avoidance Test; all the results are expresed as mean ± SEM (n = 6). [a]p< 0.001 compared to control, [b]p< 0.001 compared to vehicle, [c]p< 0.001 compared to scopolamine, [d]p< 0.001 compared to donepezil, [e]p< 0.001 compared to compound 52(5 mg), [f]p< 0.001 compared to compound 52 (10 mg). (Two-way ANOVA fallowed by Bonferroni test).



4.1.14 Mitochondrial membrane potential in brain region

Aβ can get transported in mitochondria by translocase of outer membrane ENREF 34(388) and produces ROS, that eventually cause damage to neurons and interferes mitochondrial membrane potential. The mitochondrial membrane potential was maintained in animals when treated with compounds **52** and **46** at a dose of 5 mg/kg (**Figure 4.16**).

Figure 4.16 Effect of Scopolamine induced amnesia on mitochondrial membrane potential of brain isolated from animals used in the experiment. Bars show 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; [e]p < 0.05 compared to compound 37 at dose of 10 mg/kg; [g]p < 0.05 compared to compound 46 at dose of 5 mg/kg (One-way ANOVA followed by Student Newman Keuls test).

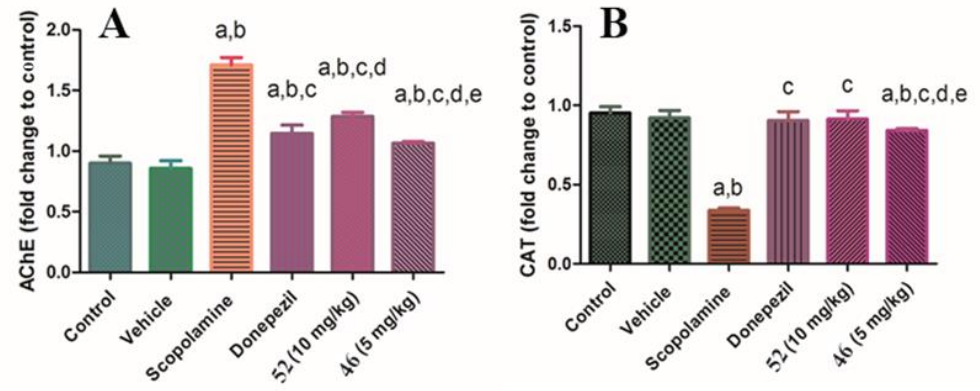


Neurochemical level estimation

The neurochemical (AChE) level in animal brain was analyzed by Ellman's method to determine the effect of treatment. Scopolamine treatment increased level of AChE, but it was significantly decreased by the treatment of donepezil and compounds **52** (10

mg/kg), **46** at 5 mg/kg. Catalase (CAT, catalyzes H_2O_2) level in brain of animals was determined after behavioral studies. Compound **52** maintained its normal level at a dose of 10 mg/kg (**Figure 4.17**). Histopathological examination of brain was carried out to determine the pattern of arrangement in normal, toxic and treated tissues. Scopolamine treated animals showed some lesions while standard pattern of tissues was found in control, compounds **52** and **46** treated animals (**Figure 4.14 I**).

Figure 4.18. (A) AChE level increased significantly in case of scopolamine and decreased substantially by donepezil and compound **46**. Bars show 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; $^{e}p < 0.05$ compared to compound **52** at dose of 10 mg/kg(One-way ANOVA followed by Student Newman Keuls test). **(B)** CAT level decreases significantly in case of scopolamine and it increases substantially by donepezil and compound **52** and **46**. Bars show 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; $^{e}p < 0.05$ compared to compound **37** at dose of 10 mg/kg.



4.3 Conclusion

Novel 3,6-diphenyl-1,4-*bis*(phenylsulfonyl)piperazine-2,5-dione derivatives were designed and synthesized by amide coupling followed by cyclization to piperazine ring. The scaffold had BBB penetrating ability along with AChE and MMP-2 inhibition potential. Compounds **38**, **39**, **46**, **53**, **54**, **55**, **47**, **56** and **50** showed promising inhibition potential against AChE while compounds **52**, **38**, **45**, **47**, **56** and **50** showed high potency against MMP-2 inhibition. Further, compounds **52** and **46** showed highest inhibition potential among all for AChE with IC_{50} = 32.45±0.044 nM, 28.65±0.029 nM, BuChE, IC_{50} = 157.95±0.264, 160.58±0.082 and MMP-2 IC_{50} = 36.83±0.015 nM, 19.57±0.005 nM, respectively. These derivatives also demonstrated critical interaction with targets in molecular modeling studies. In enzyme kinetics with AChE, lead compounds **52** and **46** showed noncompetitive inhibition and preferentially bound to the free enzyme ($\alpha > 1$), while MMP-2 was found to be inhibited competitively. Both the compounds were more potent than standard donepezil in AChE-induced Aβ aggregation study at dose of 20 μM. Neuroprotection assay of compound **46**, in MC65 cell lines indicated that it had approximately equal potency as TC for inhibition of ROS induced neurodegeneration. Metal chelation property of compound **46** was helpful in MMP-2 inhibition and AChE-induced Aβ aggregation.

The working memory assessment of compounds **52** and **46** could enhance the spontaneous alteration. Compound **52** showed a significant increase in spontaneous alteration score at 10 mg/kg, while **46** had same response at half dose (5 mg/kg) indicating that there was improvement in immediate working memory. Compound **52** reduced neophobia and anxiety at doses of 10 and 5 mg/kg as compared to scopolamine treated rats. It also displayed significant anti-anxiolytic activity which was monitored by observing the time spent by the animals in the novel arm. Compounds **52** and **46**

showed significant dose-related response in passive avoidance test. Compound **52** exhibited significant increase in TLT at 10 mg/kg while similar response was observed at half (5 mg/kg) dose of compound **46**. Further, no significant difference in the mitochondrial membrane potential of brain in treated animals was observed compared with control. The histopathological studies of brain of scopolamine treated animals revealed the complete absence of neurodegenerative lessons in treated and control groups.