CHAPTER 3

Pharmacological Evaluation of 1-[bis (4-fluorophenyl)-methyl] piperazine Derivatives for Acetylcholinesterase Inhibition in Alzheimer’s Disease
3.1 IN-VITRO ACETYLCHOLINESTERASE ASSAY

3.1.1 Introduction

The ability of a neuron to communicate with other neurons through a chemical synapse is a source of endless fascination for neuroscientists. The chemical vehicles that enable this communication are neurotransmitters. Neurotransmitters can be categorized into four groups: monoamines, amino acids, peptides, and acetylcholine. They are synthesized, packaged and transported (if necessary) to the terminal of the presynaptic cell. The arrival of the action potential at the terminal of a cell triggers the release of neurotransmitter into the synaptic cleft. After diffusion across the synapse, the neurotransmitter can activate receptors on the postsynaptic cell resulting in excitation or inhibition of that cell. Finally, the neurotransmitter is inactivated through enzymatic breakdown, re-uptake or by its diffusion. Analysis of the various biochemical events associated with synaptic transmission is the domain of the neurochemist. The concentrates on the neurotransmitter acetylcholine (ACh) and the enzyme which breaks it down, acetylcholinesterase (AChE), as an introduction to neurochemical methods.

3.1.2 Reaction Mechanism

AChE is the enzyme that degrades acetylcholine in the brain. AChE works by hydrolyzing ACh into choline and acetic acid at cholinergic synapses. The reaction in the active site between ACh and AChE occurs by the following mechanism.

Figure 1: Reaction Mechanism of AChE and ACh
The activity of AChE will be measured according to a method developed by Ellman et al. 1961. This method employs acetylthiocholine iodide (ATChI) as a synthetic substrate for AChE. ATChI is broken down to thiocholine and acetate by AChE and thiocholine is reacted with dithiobisnitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color which develops over time is a measure of the activity of AChE and can be measured using a spectrophotometer.

The activity of an enzyme is generally expressed as a rate: the quantity of substrate (in moles) which is broken down by a known amount of enzyme per unit time i.e. the amount of ATChI which is broken down by AChE per minute.

3.2 ESTIMATION OF ACETYLCHOLINESTERASE ACTIVITY (ACHE)

3.2.1 Materials and Methods

Materials

1. Spectrophotometer
2. Single edged razor blades
3. Vortex
4. Capped 25 ml (centrifuge) tubes
5. Clean or disposable 13 x 100 mm glass test tubes and test tube rack
6. Aluminum foil
7. Homogenizer
8. Pipetmen - various sizes
9. Stop watches
10. Rubber gloves
11. Rat brain
12. 0.1M phosphate buffer (PB), pH 8.0
Methods

The activity of the enzyme AChE was measured by following an increase in the absorbance produced from thiocholine when it reacts with dithiobisnitrobenzoate ion (DTNB; Ellman et al., 1961), a rapid spectrometric determination of acetylcholine esterase activity. It is based on the following reactions.

\[
\text{Acetylcholine} \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Acetate} \\
\text{Thiocholine} + \text{DTNB} \rightarrow \text{Yellow Color}
\]

Thus by this method, activity of total cholinesterase in the brain was measured. Since the activity of pseudocholinesterases is negligible compared to AChE in the brain (1:30, Ellman, et al., 1961), no specific inhibitor for pseudo cholinesterase was used.

3.2.2 Assay Procedure

Animals were decapitated and brains were removed quickly. Hippocampus of stressed and age matched rats were dissected out and weighed (Figure 2).

1. The brain tissue was homogenized (10mg/ml in 0.1M phosphate buffer at pH 8.0) in a potter Elvejem homogenizer.

2. Protein content was determined by Lowry method\(^2\) (Lowry et al., 1951) using bovine serum albumin as standard.

3. 0.4ml aliquot of homogenate was added to a cuvette containing 2.6 ml phosphate buffer (0.1M pH 8.0) and 100ul of DTNB (39.6mg DTNB with 15mg sodium bicarbonate dissolved in 100ml of 0.1M phosphate buffer at pH 7.0).

4. The contents of the cuvette were mixed thoroughly by bubbling air and absorbance was measured at 412nm in a LKB-4050 spectrophotometer. When absorbance reached a stable value, the reading was auto zeroed.

5. 20μl of the substrate in acetylthiocholine (21.67mg/ml water) was added and changes in absorbance were recorded for 10min. and the change in absorbance per minute was calculated.
6. The enzyme activity was determined using the following formula, $R = 5.74 \times 10^{-4} \times \frac{A}{Co}$, Where,

$R = \text{Rate in moles of substrate hydrolyzed per min/per gram tissue.}$

$A = \text{Changes in absorbance per min.}$

$Co = \text{Original concentration of the tissue (mg/ml)}$

Figure 2: Pictures Showing the Rat Brain Dissection
3.2.3 Inhibitor Concentration Causing 50% Inhibition of Enzyme Activity (IC$_{50}$) Values

AChE inhibition Neostigmine (a reversible cholinesterase inhibitor), was used in the concentration range 2.9 to 29 nm was used to inhibit AChE in electric eel, human serum, and rat brain homogenate. Inhibition by piperazine derivatives was studied in the presence of different concentrations of compounds and the percentage inhibition of enzyme activity was calculated. The inhibition of AChE by piperazine derivatives was analyzed with values obtained in comparison to that of neostigmine.

3.2.4 Results and Discussion

The AChE inhibition test of piperazine derivatives in rat brain is as shown in Figure 4. These derivatives exhibited 50% activity at a dose of 200, 93.75, 18.75 and 37.5 nM for compounds 9a, 9b, 9c and 9h respectively. The significant potency of inhibition was shown by 9c followed by 9h, 9b, and 9a in increasing order.

Nevertheless the AChE inhibition test of alkyl and aryl sulfonyl piperazine derivatives in rat brain is as shown Figure-7. These derivatives exhibited 50% activity at a dose of 208, 98.65, 21.62 and 37.5 nM for compounds 10c, 10d, 10g and 10h respectively. The potency of inhibition is highest in 9c followed by 9h, 9b, 9a and 10d followed by 10c, 10g, and 10h in increasing order (Figure 3).

The pharmacological conclusion for compounds 9a, 9b, 9c, 9h, 10c, 10d, 10g and 10h is anticholinesterase is conformed by the biochemical finding on rat brain, human serum and electric eel AChE. Most of the compounds tested did not show any inhibitory activity. The compounds compress well with the standard Neostigmine. Compound 9c and 10d is the most potent of all the inhibitory compounds tested. This leads to suggest that compounds like 9c and 10d offer further modification can lead to the development of a potent AChE inhibitor that can find a place in the treatment of AD.

The inhibitory activity of the newly synthesized compounds against AChE was studied using the method of Ellman et al.$^3$ to determine the rate of hydrolysis of acetylthiocholine iodide in the presence of the inhibitor against different sources of
AChE, rat brain homogenate AChE, electric eel AChE and human serum AChE that are as shown in Figure 4–9 respectively.⁴

Activities of the synthesized compounds were compared with the inhibitory activity shown by the known standard inhibitor neostigmine. Different derivatives of 1-[bis(4-fluorophenyl)-methyl]piperazine having different heterocyclic rings were tested for their ability to block the AChE activity for the substrate acetylthiocholine iodide. The order of potency is 9c > 9h > 9b > 9a. The other compounds screened failed to elicit any inhibition of acetylcholinesterase from rat brain homogenate. Among the molecules screened for the AChE inhibitory activity, pyrrolidine substituted piperazine 9c (IC₅₀ = 12.4, 17.6, and 18.6 nM) was found to effectively block the enzyme as compared to the rest of the derivatives studied. Piperidinyl (9b) and morpholinyl (9a) derivatives are also effective in blocking the AChE enzyme activity (IC₅₀ = 93.75, 126.3, 135.2, 200, 284.2 and 252.9 nM), respectively (Table-1). The 2-cyanobiphenyl ring also shows good activity probably because of its bulkiness.

Among the sulfonyl piperazine derivatives of 1-[bis (4-fluorophenol)-methyl]piperazine (10a-j) are tested for their ability to block the AChE activity for the substrate acetylthiocholine iodide. In general sulfonamides are effective probably because of sulfonyl linkage as supported by many active sulfonamide drugs. In addition cinnarizine, a well-known cerebral vasodilator, passes through the blood–brain barrier (BBB)⁵ because of the presence of lipophilic group (diphenyl methyl piperazine moiety) in the molecule. Among the compounds (10a-j) screened for the AChE inhibitory activity, methane sulfonyl chloride substituted piperazine 10d (IC₅₀= 17.5, 22.8, 24.5) showed better effective block in the enzyme in a better way than the rest of the compounds. 4-Nitro-benzene sulfonyl chloride (10c) and 2,5-dichlorobenzene sulfonyl chloride (10h) derivatives were also effective in the blocking the AChE activity. (IC₅₀ =105, 120, 102.8, 264.6, 255, 246 µM). On the basis of previous information compounds (10a-j) synthesized herein can be expected to enter the central nervous system (CNS) because of the structural similarity with cinnarizine. According to recent studies piperazine analogues inhibit AChE the electron donating effect is the most important factor on the benzyl benzene ring, suggesting a role in regulating the protonation equilibrium at the benzylic nitrogen of the piperazine skeleton. The smaller the substituent, the more favorable activity at diphenyl methyl site of the molecule. The
aromatic fluoro substituent is slightly smaller than hydrogen in terms of molecular refractivity (MR), being 0.09 versus 0.106.

Figure 3: The Most Significant Molecules on AChEIs
3.2.5 Conclusion

The piperazine derivatives 9c and 10d are found to be potent inhibitors of AChE. In addition, 9h, 9b, 9a, 10g, 10h and 10c found to be selective and significant inhibitors of AChE in vitro. The present study reveals that the synthesized compounds 9c, 9h, 9b, 9a, 10d, 10g, 10h and 10c showed significant inhibition (Figure 3) against cholinesterase, which were obtained from different sources. Therefore, they could be used as biochemical tools for the development of new drugs for Alzheimer’s disease.
Table 1: Comparative Inhibitory Activities of 1-[bis (4-fluorophenyl) methyl] piperazine Derivatives 9(a-h) and 10(a-j) Against AChE from Different Sources.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Rat Brain Homogenate</th>
<th>Human Serum</th>
<th>Electric eel</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>IC$_{50}$ (nM)</td>
<td>IC$_{50}$ (nM)</td>
<td></td>
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<tr>
<td>9a</td>
<td>200</td>
<td>284.2</td>
<td>252.9</td>
<td></td>
</tr>
<tr>
<td>9b</td>
<td>93.75</td>
<td>126.3</td>
<td>135.2</td>
<td></td>
</tr>
<tr>
<td>9c</td>
<td>18.75</td>
<td>21.0</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>9d</td>
<td>NI</td>
<td>3203.7</td>
<td>2127.4</td>
<td></td>
</tr>
<tr>
<td>9e</td>
<td>NI</td>
<td>1072.8</td>
<td>1210.2</td>
<td></td>
</tr>
<tr>
<td>9f</td>
<td>NI</td>
<td>1341.7</td>
<td>1136.2</td>
<td></td>
</tr>
<tr>
<td>9g</td>
<td>NI</td>
<td>1012.9</td>
<td>715</td>
<td></td>
</tr>
<tr>
<td>9h</td>
<td>37.5</td>
<td>73.6</td>
<td>70.5</td>
<td></td>
</tr>
<tr>
<td>10a</td>
<td>208</td>
<td>98.65</td>
<td>21.62</td>
<td></td>
</tr>
<tr>
<td>10b</td>
<td>NI</td>
<td>2453.5</td>
<td>2734.4</td>
<td></td>
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<td>10d</td>
<td>17.5</td>
<td>22.5</td>
<td>24.5</td>
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</tr>
<tr>
<td>10e</td>
<td>NI</td>
<td>1345.2</td>
<td>2943.3</td>
<td></td>
</tr>
<tr>
<td>10f</td>
<td>NI</td>
<td>1004.6</td>
<td>1184.7</td>
<td></td>
</tr>
<tr>
<td>10g</td>
<td>83.7</td>
<td>123.5</td>
<td>138.4</td>
<td></td>
</tr>
<tr>
<td>10h</td>
<td>96.8</td>
<td>134.9</td>
<td>143.2</td>
<td></td>
</tr>
<tr>
<td>10i</td>
<td>NI</td>
<td>993.6</td>
<td>1203.8</td>
<td></td>
</tr>
<tr>
<td>10j</td>
<td>NI</td>
<td>1423.4</td>
<td>1534.5</td>
<td></td>
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<tr>
<td>Neostigmine</td>
<td>37.5</td>
<td>42.1</td>
<td>41.1</td>
<td></td>
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</table>

NI- No inhibition found

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Figure 4: Concentration Dependent Inhibition of Rat Brain Homogenate AChE by the Significant Molecules.

![Graph showing concentration-dependent inhibition of Rat brain homogenate AChE by piperazine derivatives.]

Figure 5: Concentration Dependent Inhibition of Electric eel AChE by the Significant Molecules.

![Graph showing concentration-dependent inhibition of Electric eel AChE by piperazine derivatives.]

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Figure 6: Concentration Dependent Inhibition of Human Serum AChE by the Significant Molecules.

![Graph showing concentration dependent inhibition of human serum AChE by piperazine derivatives](image)

Figure 7: Concentration Dependent Inhibition of Rat Brain Homogenate AChE by the Significant Molecules.

![Graph showing concentration dependent inhibition of rat brain homogenate AChE by piperazine derivatives](image)
Figure 8: Concentration Dependent Inhibition of Electric eel AChE by the Significant Molecules.

![Inhibition of electric eel AChE by piperazine derivatives](image1)

Figure 9: Concentration Dependent Inhibition of Human Serum AChE by the Significant Molecules.

![Inhibition of human serum AChE by piperazine derivatives](image2)
3.3 IN VIVO CHOLINESTERASE ASSAY

Antiamaemic effect of 1-[bis(4-flurophenyl)methyl]piperazine derivatives against scopolamine induced passive avoidance step-down task paradigm in rats.

3.3.1 Introduction

Passive-avoidance response (PAR) is extensively used for the screening of drugs affecting learning and memory\textsuperscript{7-13}. The test involves training rodents to avoid punishment (normally an electric shock) by curbing a normal behavior (such as the exploratory behavior). At specified intervals after training, the animals are tested again for retention of such learning. The conventional and the most widely employed parameters are step-down latency (SDL) and step-down errors (SDE).

3.3.2 Scopolamine

Scopolamine acts by interfering with the transmission of nerve impulses by acetylcholine in the parasympathetic nervous system and produces symptoms typical of parasympathetic system depression: dilated pupils, rapid heart beat and dry skin, mouth, and respiratory passages. Because scopolamine depresses the central nervous system, it is used as a sedative prior to anesthesia and as an antispasmodic in certain disorders characterized by restlessness and agitation, e.g., delirium tremens, psychosis, mania, and Parkinsonism. When combined with morphine, the effect produced is a tranquilized state known as twilight sleep; this combination of drugs was formerly used in obstetrics but is now considered too dangerous. Over dosage of scopolamine causes delirium, delusions, paralysis, and stupor. The alkaloid is found in a variety of nonprescription sedatives.

3.3.3 Passive-Avoidance Response:

The methods described by Papazova et al. and Kulkarni and Verma were suitably modified. A continuous avoidance response apparatus (Techno, Lucknow) with an inverted Petri dish as the shock-free zone (SFZ) was used. The test mouse was placed on the SFZ located in the center of the wire grid floor of the chamber. When the animal stepped off this SFZ, it received a shock (20 volts). The mice were given 3-5 trials and trained to avoid punishment (remain on SFZ) for at least 60 seconds. Animals not meeting these criteria of learning in 5 trials were rejected. Retention parameters
(SDL, SDE and TSZ) were noted for 10 min, 4hr after training and compared for different treatments (Figure 10).

![Passive Avoidance Apparatus](image)

**Figure 10: Passive Avoidance Apparatus**

### 3.3.4 Materials and Methods

#### Materials

1. Distilled water
2. Animals Rat (150-200 g)
3. Drugs: Scopolamine (0.4, mg/kg, ip)

#### Method

1. Turned on the equipment and switched on the latency mode.

2. Experimentally animals are grouped as control, scopolamine treated and scopolamine with test compounds treated groups, each comprising of 6 animals. Weighing between 150-200g. Male and female animals are experimentally used in same number in each group.

3. Dose response curve was plotted by treating one of the experimental compounds (9a and 10d) in five different dose and was found 0.1mg/kg body weight shows maximum activity.
4. Each rat was placed individually on the electric grid and allowed it to explore the apparatus for about one minute.

5. The start button pressed after selecting proper voltage current (20 V) for foot shock. Pressed start button till the animal reaches centrally located shock free zone (SFZ). Noted the latency (in seconds) as displayed in the timer. And rejected the animals, which take more than 2 minute to reach SFZ.

6. Placed the animal back on electric grid and repeated the step 3 to get at least three basal readings so that the animal gets acquainted with the task trained.

7. After one hour of the training, placed the animal on the electric grid one more time and repeated step 3. The latency noted.

8. Turned on the mistake mode, adjusted timer to 15, pressed start button Counted time on the display and mistakes as soon as the animal touched the electric grid and got shock. the mistakes that the animal made in 15 minutes were noted.

9. To another group after step 4, i.e., in trained rat administered the dose of 0.4mg/kg scopolamine by ip. After 30 minutes step 5-6 were repeated.

10. Compared the latency in reaching SFZ and number of mistakes in 15 minutes by scopolamine treated animal group and scopolamine with compounds treated groups (Table 2 and Figure 11).
Table 2: Study of Antiarnnesic Effect of 1-[bis (4-fluorophenyl) methyl] piperazine Derivatives 9(a-h) and 10(a-j) against Scopolamine Induced Memory Loss.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Treatment (dose)</th>
<th>Basal Latency (sec) of Rat to Reach Shock Free Zone (SFZ)</th>
<th>Memory Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dose) mg/kg. i.p</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Control Groups</td>
<td>-</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Scopolamine Treated Groups</td>
<td>0.4</td>
<td>36</td>
<td>10</td>
</tr>
<tr>
<td>9a Treated Groups</td>
<td>0.1</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>9b</td>
<td>0.1</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>9c</td>
<td>0.1</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>9d</td>
<td>0.1</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>9e</td>
<td>0.1</td>
<td>35</td>
<td>9</td>
</tr>
<tr>
<td>9f</td>
<td>0.1</td>
<td>36</td>
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<tr>
<td>9g</td>
<td>0.1</td>
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<tr>
<td>9h</td>
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<td>7</td>
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<tr>
<td>10a</td>
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<td>10b</td>
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<tr>
<td>10c</td>
<td>0.1</td>
<td>23</td>
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<tr>
<td>10e</td>
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<tr>
<td>10f</td>
<td>0.1</td>
<td>33</td>
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<td>0.1</td>
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<td>10h</td>
<td>0.1</td>
<td>24</td>
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<tr>
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<td>8</td>
</tr>
<tr>
<td>10j</td>
<td>0.1</td>
<td>20</td>
<td>6</td>
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</table>
3.3.5 Results and Discussion

Scopolamine significantly increase (p<0.01) the latency to reach the SFZ and the number of mistakes when compared to control group. From the SAR, it is observed that the piperazine basic ring containing single or non-fused heterocyclic moieties (R = 9a, 9b, 9c, 10g, 10h and 10d) showed a better activity than fused heterocyclic moieties (R = 9d, 9e, 9f, 10a, 10b, 10g and 10i). It is also observed from reversing amnesic effect of scopolamine induced memory loss in passive avoidance step-down task paradigm in rat. Compound 9c and 10d reverses the average number of mistakes done from 35 (scopolamine) to 10. Values for all the compounds are given in Table 2, which show that in vivo and in vitro results are fairly comparable.

It may be concluded from this study that, for effective binding and blocking of the AChE activity, molecule bind with the peripheral site and the active site of the enzyme and it may be possible that the piperazine binds to the active site and the substituents containing heterocyclic rings (9a–h) and (10a–j) bind to peripheral site of the enzyme. Therefore, it can be summarized that substitution of other heterocyclic
rings on piperazine basic nucleus separated by two carbons needs to be studied for better AChE inhibitory activity.

3.3.6 Future Prospects

The general acceptance of the cholinergic hypothesis of AD has experienced some ups and downs due to the modest effects displayed by AChEIs and the failure of the other classes of cholinomimetics. However, the finding that APP processing may be under cholinergic control, and therefore, the assumption that cholinergic pharmacotherapy might have more than a symptomatic role in the management of AD, has boosted a recent revival of this strategy. While AChEIs are likely to be actively used for the indefinite future as the only available efficacious treatment for AD.

The development of allosteric modulators of both muscarinic\textsuperscript{15} and nicotine\textsuperscript{16} receptors, as a way to achieve better subtype selectivity and to prevent compensatory processes such as receptor desensitization or down regulation of expression, which are induced by agonists. On the other hand, treatment of AD with a single drug is probably not a realistic option due to the complicated nature of the disease. In this sense, a combination therapy of AChEIs with muscarinic or nicotinic agonists or with noncholinergic agents such as estrogens, antioxidants or anti-inflammatory agents will be a future alternative to the present monotherapy\textsuperscript{17}. Moreover, combination therapies could be directed to the manipulation of several neurotransmitters, which are deficient in AD patients and are also involved in the various components of memory and the cognitive ability. Recent evidence suggests that combined cholinergic-monoaminergic therapies are markedly effective in restoring some aspects of cortical functioning\textsuperscript{18}, and a combination therapy of AChEIs with the N-methyl-D-aspartate (NMDA).
REFERENCES


