CHAPTER - 5

Pharmacological Screening of Thiazolidin-4-one Arecoline Derivatives for M1 Receptor Binding Studies
5.1 INTRODUCTION

Muscarinic receptors belong to the super family of G protein-coupled receptors, and mediate many of the actions of acetylcholine throughout the brain and peripheral tissues. Five muscarinic receptor subtypes have been identified and characterized using a variety of pharmacological and molecular biological techniques.\(^1\) Several lines of evidence have suggested that selective muscarinic agonists could be useful in the treatment of schizophrenia. Anticholinergic drugs, including the glycolate esters which produce psychotomimetic effects in humans.\(^2\) High doses of muscarinic antagonists used in the treatment of Parkinson’s disease (e.g., benztpine, trihexyphenidyl) produce hallucinations and confusion. In contrast, Alzheimer’s disease patients treated with cholinesterase inhibitors, which elevate levels of acetylcholine, exhibit improvements in neuropsychiatric symptoms such as agitation, hallucinations, and psychosis.\(^3\)-\(^5\) In addition, the selective M1 muscarinic agonist xanomeline significantly improved psychiatric symptoms such as hallucinations in phase II clinical trials in Alzheimer’s patients.\(^6\)

Muscarinic agonists might be useful in the treatment of Alzheimer’s disease neurological disorders, including, schizophrenia, chronic pain, and drug abuse. Previous studies identified a series thiazolidin-4-one arecoline derivatives with high activity and selectivity for muscarinic receptors. To develop compounds with improved central nervous system penetration, several new derivatives were synthesized and characterized for muscarinic receptor binding and activity.

5.1.1 Radioligand Binding

Many biochemical processes, essential for the functioning and survival of cells (and the organism), are regulated by hormones, neurotransmitters, cytokines and other “messenger” molecules. This regulation proceeds by interaction of these naturally occurring molecules with receptors that are either embedded in the cell membrane (membrane-bound) or present in the cytoplasm (soluble receptor) or the nucleus of the cell. The membrane-bound receptors can be subdivided into G-protein coupled receptors (GPCRs), ion channels and receptors with a single transmembrane segment. GPCRs interact with GTP binding proteins and consist of seven-transmembrane
helices. Ion channels are homo- or hetero-oligomeric receptors that are composed of several subunits arranged in a ring that forms the ion channel containing the ligand-binding sites. Nuclear or soluble receptors are represented by the group of steroid receptors (e.g. the estrogen receptor) and the non-steroidal receptors (e.g. Vitamin D receptor) that regulate biological functions by controlling gene expression. This class of receptors consists of a DNA-binding and a ligand-binding domain. Changes in receptor density and a disturbed balance in the (in) activation of these receptors give rise to the development of disease. For example, Parkinson’s disease is related to a change in dopamine D2 receptor density\(^7\). Because of the involvement of these receptors in disease development, they are important targets in drug discovery. It is thus not surprising that, for example, drugs that interact with G-protein coupled receptors make up 50% of all available therapeutics\(^8\)–\(^9\) and are therefore of major interest. Receptor screening methodologies can be based on either the determination of a functional response (e.g. cell proliferation), the production of second messengers (e.g. Ca\(^{2+}\)) or the interaction of a ligand with its receptor\(^10\). With respect to functional assays, it is possible to simultaneously monitor several signaling events such as enzyme activation (e.g. adenylate cyclase) and the mobilization of Ca\(^{2+}\), and to differentiate between agonistic and antagonistic properties. There is a trend towards the development of cell-based assays (e.g. to replace animal studies), which has been facilitated by recombinant DNA technology using reporter gene systems. Nevertheless, it is sometimes still costly and difficult to obtain stable eukaryotic cell lines\(^11\). Binding of a ligand (agonist or antagonist) to its cognate receptor is the initial and indispensable step in the cascade of reactions that finally cause a pharmacological effect\(^10\) and many successful and widely used techniques are thus based on measuring ligand binding. Various assay formats to measure the interaction of a ligand with its receptor will be covered in this review, but many of the detection technologies discussed here are also applicable to cell-based assays\(^11\). Description of the different technologies will be accompanied by an overview of labels that can be utilized in receptor binding assays.

A radioligand is a radioactively labeled drug that can associate with a receptor, transporter, enzyme, or any site of interest. Measuring the rate and extent of binding provides information on the number of binding sites, and their affinity and accessibility for various drugs\(^12\)–\(^13\).
5.1.2 Receptor–Ligand Binding Assay Technologies

Receptor–ligand binding assays may be classified according to the need for separation of bound from free ligand or the detection technique. Indexed by the first criterion, the assay types are heterogeneous, homogeneous and non-separating homogeneous. Heterogeneous assays require separation of the free from the bound fraction of the ligand by filtration, centrifugation or dialysis before measurement. A homogeneous assay requires no separation or washing steps before measurement, resulting in the development of the so called mix-and-measure or mix-and-read assays, which is often an advantage when it comes to assay automation and miniaturization. In the non-separating homogeneous assay, the signal is centered on or around a solid phase which contains the immobilized receptor or ligand. In this assay format, there is also no need to physically separate the free from the bound fraction. The ideal assay should be specific, sensitive, easy to perform, reliable and reproducible, cheap, rapid and suitable for automation. Furthermore, there is a preference for non-radioactive assay formats to reduce health risks and environmental pollution as well as costs. Next to this, the possibility to quantify multiple analytes in a single assay becomes more and more important, so the system should preferably be capable of multiplexing. Next to the formats mentioned above, it is possible to determine the ligand binding properties via affinity chromatography using immobilized receptors and interfacial optical assays, e.g. total internal reflection fluorescence (TIRF) and surface plasmon resonance (SPR).

5.1.3 Liquid Scintillation Counters (LSC)

LSC are instruments commonly used to detect radioisotopes that emit low energy β-particles. A sample with an unknown amount of a radioisotope is placed into an organic or aqueous solution. This solution, commonly called the “counting cocktail” causes the radioisotope to emit small flashes of light. These flashes are detected and converted to amplified electrical pulses by a photomultiplier tube. Liquid scintillation counters can distinguish between different isotopes and different energy types emitted by an isotope.

In general, LSC carry out the following functions:
1. Sense light flashes from the radioisotope and converts this energy to voltages that are proportional to the intensity of the light flash.

2. Sort through these voltages and put them into energy ranges.

3. Count the number of voltages in each energy category.

5.1.4 Applications of the Liquid Scintillation Counters Technique

LSC can be used to detect radioisotopes in any liquid sample. This includes blood, urine, cytosol, or any other homogenous liquid. This method can be useful in the biological sciences as well as in traditional chemistry. The following list includes some of the ways liquid scintillation Counters can be used:

1. For safety inspections. Liquid scintillation counters are extremely sensitive, and can detect radioactivity below the detection limits of traditional Geiger Counters.

2. To track radioisotopes as they are digested in an animal or cell culture. This quantitatively determines the extent by which a specific nutrient/molecule is metabolized.

3. Quantifying genetic material (DNA and RNA) with radioactive nucleotides.

4. More generally, any experiment involving radioactivity can use a liquid scintillation counter in some way.
1. Add cells, membrane, or tissue to Multi screen Plate
2. Incubate with labeled ligand
3. Collect receptor/ligand complex on filter membrane. Wash and count. If “free Vs. bound” study, collect filtrate into 96-well plate

Figure 1: The Equipments Used for the M1 Receptor Binding Studies
5.1.5 The Role of the Solvent (Cocktail)

The solvent portion of an LSC cocktail comprises from 60-99% of the total solution. When a radioisotope dissolved in the cocktail undergoes an emission event, it is highly probable that the particle or ray will encounter only solvent molecules before its energy is spent. For this reason, the solvent must act as an efficient collector of energy, and it must conduct that energy to the phosphor molecules instead of dissipating the energy by some other mechanism. The solvent must not quench the scintillation of the phosphor, and, finally, the solvent must dissolve the phosphor to produce a stable, countable solution. Aromatic organics have proven to be the best solvents for LSC. The prototypical LSC solvent is toluene (The solvents used in National diagnostics scintillation fluids are safer and less toxic than toluene). The π cloud of the toluene ring (or any aromatic ring) provides a target for β-interaction, which captures the energy of the incident particle. This captured energy is generally lost through transfer to another solvent molecule, as toluene has little tendency to emit light or undergo other alternate decay modes. Thus, a β-particle passing through a toluene solution leaves in its wake a number of energized toluene molecules. The energy from these molecules passes back and forth among the solvent ring systems, allowing efficient capture by dissolved phosphors.

5.2 ESTIMATION OF DENSITY AND AFFINITY OF RECEPTOR

5.2.1 Principle

The direct radioligand binding assay measures the direct interaction radioligand with the receptor. In this assay, the increasing concentration of radioligand is allowed to interact with tissue preparation containing receptors in presence and absence of cold displacer to block the non-specific binding of radioligand. This technique is used to estimate the density and affinity of receptor.

The basic ligand-binding model involves the reversible interactions of ligand [L] with a single class of receptor [R] following the law of mass action.

\[ k_1 \quad [R] + [L] \rightleftharpoons \quad k_2 \quad [RL] \]
Where, \( k_1 \) and \( k_2 \) are rate constants for association and dissociation, respectively. Thus at equilibrium,

\[
K_d = \frac{k_1 \left[ R \right] [L]}{k_2 [RL]}
\]

Where,

\[ [R] = \text{concentration of receptor}, \]
\[ [L] = \text{concentration of ligand and} \]
\[ [RL] = \text{concentration of receptor ligand complex}. \]

The equation is set up according to the dissociation of receptor-ligand complex \([RL]\) and the ratio \( k_1/k_2 \) is defined as the dissociation constant \((K_d)\). Assuming that a finite number of receptors exist, defined as \( B_{\text{max}} \), it follows that,

\[ [R] + [RL] = B_{\text{max}} \]

And hence the relationship can be derived as.

\[
[B_{\text{max}} [L]]/[1 + K_d/[L]]
\]

Where, \([RL]\) = the amount of ligand bound to receptor (B). Thus

\[
B = \frac{B_{\text{max}} [L]}{K_d + [L]}
\]

In the basic receptor model, the binding site population us defined by two parameters, the dissociation constant \((K_d)\) and the maximal binding sites \((B_{\text{max}})\). The above expression is analogous to Michaelis Menton equation of enzyme substrate interaction, where \( K_d \) is equivalent to \( K_m \). The \( K_d \) is frequently used as a measure of affinity of interaction between ligand and receptor and is expressed in units of concentration (nM). The lower the \( K_d \) value, higher is the affinity of interaction and vice versa.
5.2.2 Scatchard Plot

A plot of the ratio of bound to free radioligand against the bound is called scatchard plot. Two parameters, B_max and K_d values can be calculated from this plot. The intercept on the x-axis indicates B_max, where as the reciprocal of slope of line give the K_d value.

An advantage of using Scatchard analysis is that to provide an estimate of the total concentration of receptors without requiring saturating concentration of radioligand. Thus the concentration of receptor can be estimated by extrapolating a straight line to the abscissa. This is particularly important in systems with high levels of non-specific binding. Another advantage of scatchard plot is that the visual inspection provides insight into whether or not a simple bimolecular reaction adequately describes the interaction between the ligand and receptor. Curvature of the scatchard plot represents the complex interaction. A scatchard plot that is concave upward can result from heterogeneous population of receptors or negative cooperatively between the binding sites. A Scatchard plot that is concave downward can result from positive cooperatively between the binding sites or from failure of the reaction to reach the equilibrium at lower concentration of the ligand, since the time to reach equilibrium is a function of ligand concentration.

5.2.3 Hills Plot

A further linear transformation of saturation data is the Hills plot. This analysis has the advantage of providing additional information one the mode of interaction between receptor and ligand. Hills plot occupies a central position in ligand binding studies where there is more than one apparent number of binding sites. If ‘n’ is the number of apparent binding sites on a receptor, then the reaction can be described by,

\[ [R] + n[L] = n[RL] \]

The binding isotherm is then expressed as,

\[ B = \frac{B_{\text{max}} \times n[L]}{K_d + n[L]} \]
This is Hill’s equation and may be transformed to,

\[ \log B = \frac{n \log [L] - \log K_d}{(B_{max} - B)} \]

When \( \log B/(B_{max} - B) \) is plotted against \( \log [L] \), the slope of the line equals the apparent stoichiometry of the interaction. Hill coefficient value more than one indicates interaction of the ligand with more than one site, whereas Hill coefficient value indicates binding of ligand to one class of receptors.

5.2.4 Determination of Chemical Concentration of Radioligand

The chemical concentration can be determined from the radioactive concentration and specific activity by using the following formula.

\[
\text{Chemical concentration} = \frac{\text{Radioactive Concentration (Ci/ml)}}{\text{Radioactive Concentration (Ci/ml)}}
\]

\[
= \frac{\text{Radioactive Concentration (Ci/ml)}}{\text{Specific activity (Ci/mmol)}}
\]

The following equation can be used to calculate the radiochemical concentration in terms of micrograms.

Where,

\[ W = A (\mu \text{Ci}) (t^{1/2}) (C) \]

\( W \) = Weights in micrograms

\( A \) = Atomic mass

\( \mu \text{Ci} \) = Number of micro curies

\( t^{1/2} \) = Half life (\( ^3\text{H} \)12.3 years)

\( C \) = Constant (Years \( 2.80 \times 10^{-6} \))
Radioligands obtained commercially were diluted in assay buffer to obtain the required concentrations by determining their chemical concentration using the above equations.

### 5.2.5 Scatchard Analysis Quantities the Receptor Ligand Interaction

Receptor ligand binding is described by the equation

$$
R + L \rightarrow RL
$$

where 

- $R$ = Receptor 
- $L$ = Ligand 
- $RL$ = Receptor Ligand complex

This binding, depend upon the concentration of ligand and can be described by equilibrium constant.

$$
\frac{K+1}{RL} = \frac{Ka}{[R][L]} = \frac{1}{K_d}
$$

Where,

- $Ka$ = association constant
- $K_d$ = dissociation constant

Receptor ligand binding is saturable. As more ligand is added to a fixed amount of receptor, an increasing fraction of receptor molecules is occupied by ligand.

A rough measure of receptor binding affinity is given by the concentration of ligand needed to give half saturation with receptor.

A rough measure of receptor binding affinity is given by the concentration of ligand needed to give half saturation of the receptor.

Scatchard analysis: Receptor ligand binding, we can estimate both the dissociation constant ($K_d$) and the number of receptor binding sites in a given synoptosomal membrane protein preparation.
When binding has reached equilibrium (saturated), the total number of possible drug sites \([B_{\text{max}}]\) equals to the number of unoccupied sites \([R]\) number of occupied sites \([R_L]\)

\[
B_{\text{max}} = [R] + [R_L] \quad \text{(4)}
\]

The number of unbound sites can be expressed in terms of total sites – occupied.

\[
[R] = B_{\text{max}} - [R_L] \quad \text{(5)}
\]

Equilibrium expression can now be written

\[
K_a = \frac{[R_L]}{[L][B_{\text{max}} - R_L]} \quad \text{(6)}
\]

Rearranging to obtain the ratio of receptor bound ligand to free ligand (unbound)

\[
\frac{[R_L]}{[L]} \quad \frac{\text{Bound}}{\text{Free}} = \frac{\text{Bound}}{\text{Free}} = \frac{K_a[B_{\text{max}}-R_L]}{[L]} \quad \text{(7)}
\]

Bound
\[
\frac{[R_L]}{[L]} \quad \frac{\text{Bound}}{\text{Free}} = 1/ K_d[B_{\text{max}}-R_L] \quad \text{(8)}
\]

From this slop intercept from the equation is

We can see that a plot of Slope and intercept were plotted from the equation,

\[
\frac{[R_L]}{[L]} \quad \frac{\text{Bound}}{\text{Free}} \quad [\text{Bound} (RL) / [L] \text{ vs Bound (RL) } \text{ (9)}
\]

\[
\frac{[L]}{-1} \quad \text{Should give a straight line with a slope of } \frac{K_a}{K_d}
\]
5.2.6 Data Analysis

The data obtained from the binding experiments were analyzed using computer assisted 'LIGAND' program (McPherson, 1983). The Scatchard analysis gives an estimation of receptor density or maximal binding sites (Bmax) and the apparent dissociation constant (Kd) of the receptor. The "LIGAND-DRUG" programme was used to analyze the drug displacement data to obtain the inhibition constant (Ki) and the concentration of the drug required for the inhibition of the ligand binding to the receptor by 50% (IC50). The Bmax and Kd values were expressed as mol/mg protein and nM, respectively. The values of Ki and IC50 were expressed in nM.

5.2.7 Materials and Methods

1. [3H]QNB (s.a. 48.0 Ci/mmol)
2. Rat Brain (Adult male Sprague-Dawley rats, weighing 200-250 gm)
3. Phosphate assay buffer, pH 7.4 (50mM KH2PO4 and Na2HPO4 + 1mM MgCl2)
4. Tris buffer, pH 7.4 (50mM)
5. Scintillation counter
6. Millipore multiscreen plate
7. Scintillation fluid
8. Atropine (Sigma Aldrich)
9. Arecoline
10. Scintillation vials
11. Multi screen Punching Machine
12. Vacuum Box
13. Thiazolidin-4-one derivatives
5.3 PHARMACOLOGICAL CHARACTERIZATION OF M1 RECEPTOR IN RAT BRAIN

Male Sprague Downey rats weighing 200-250 gm were sacrificed by decapitation, brain was immediately removed and cerebral cortex was dissected out, cortical membranes were prepared according to the method of crease and snyder (1978).

The cortical tissue was homogenized in 20 volumes of sucrose buffer (0.32 M; pH 7.4). This tissue homogenate was centrifuged at a speed of 1000g for 10 minutes at 4°C. Resultant pellet was discarded and supernatant was centrifuged at 32,000g for 20 mins at 4°C. Pellet obtained was resuspended in Tris buffer (50 mM; pH 7.4) and incubated at 37°C for 10 min, in order to allow catabolism of the endogenous agonists. The incubated membrane pellet was made up to the level of the original supernatant volume with Tris buffer (50 mM; pH 7.4) and recentrifuged at 32,000g for 20 minutes at 4°C. The final pellet containing crude membrane was suspended in Phosphate assay buffer (50mM KH2PO4 and Na2HPO4 + 1mMgCl2; pH 7.4). Protein content of this membrane was estimated by Lowry's method (1951) and used for the binding studies.

5.3.1 Protocol for M1 Receptor Binding Assay

The M1 receptors were labeled using [3H]QNB (M1 receptor antagonist) in cortical membranes, obtained from rat brains following essentially the method described by Hyttel et al, (1992), with minor modifications. The optimal conditions for binding experiments were obtained by varying each parameter at a time of conduction the binding experiments. Experiments were done with different concentration of [3H] QNB incubated with different amounts of membrane protein (50-100 μg), at different incubation times (30, 60, 90, 120, 150, 180 mins), and at different temperatures (25, 30, 37 and 45°C). Specific binding was obtained by using different concentration of displacer (10, 50, 100, 500, 1,000 and 10,000 nM Atropine). From these experiments; conditions for optimal binding were obtained. By using these optimal conditions the reaction for binding was run by incubating 50 μg of membrane protein with 0.04-0.4 nM of [3H] QNB at 37°C for 2 hrs in a 96 well Millipore GF/B micro plate. 100nM atropine was found to be optimal for defining non-specific binding. The reaction volume was made up to 200 μl with Phosphate assay buffer (50mM KH2PO4 and
Na₂HPO₄ + 1 mM MgCl₂; pH 7.4). The reaction was stopped by the addition of 50 μl of ice-cold assay buffer and the reaction mixture was rapidly filtered through Millipore multiscreen filtration unit to separate the bound and unbound radioligand under vacuum. This was followed by a 200 μl wash, using ice-cold assay buffer. Filters were dried at 60°C for 1 hour and punched into the scintillation vials. Then 5 ml of scintillation cocktail was added and the radioactivity was counted after overnight equilibration (12-16 hrs). The vials were labeled and placed in separate racks and radioactivity was measured in a Tril-Carb 2100TR liquid scintillation analyzer (M/s. Packard Instrument Co. USA), with an efficiency of 65%.

The density of M1 receptor was 480±36 fmol/mg protein. The Kₐ values in cortex were 0.10±0.02 nM. The Hill coefficient values in cortex were near to unity, suggesting that the radioligand binds to a single component of binding sites at the concentrations used in the experiments (Figure-2).

5.3.2 Drug Inhibition Studies

Drug inhibition study, an indirect binding assay, measures the inhibition of radioligand binding to the receptors by the drugs (unlabeled ligand) to deduce indirectly the affinity of receptor for unlabeled ligand/drug. Competitive inhibition involves the interaction of unlabeled ligands with a receptor by displacing the labeled radioligand. At a particular concentration, cold ligand or drug displaces the bound radioligand from the receptor. From this assay, two important parameters can be generated, such as, inhibition constant (Kᵢ) and the concentration of inhibitor required for the inhibition of 50% of radioligand binding (IC₅₀). Based on the Kᵢ values, it is possible to determine the affinity of pharmacological agents towards a specific receptor subtypes.

The simplest model describing the interaction of a radioligand [L] and a competitive inhibitor [I] with a receptor is,

\[
\begin{align*}
[L] + [I] + [R] & \xrightarrow{k_1} [LR] + [IR] \ldots \ldots \ldots \ldots \ldots [1] \\
[1] & \xrightarrow{k_2} [B_i] \ldots \ldots \ldots \ldots \ldots [2]
\end{align*}
\]
Where $[Bi]$ is the concentration of receptor occupied by the inhibitor at equilibrium,

$$B_{\text{max}} [I] [B] = [I] + Ki (1 + (L)/Kb) \ldots \ldots \ldots (3)$$

Where, $Ki$ is the equilibrium dissociation constant of the competitive inhibitor. If $K_d$ is much greater than the concentration of receptor in the assay, the fraction of receptor occupied by inhibitor ‘f’ is

$$[Bi] = [I] f = B_{\text{max}} [I] + IC_{50} \ldots \ldots \ldots (4)$$

In this equation, $IC_{50}$ is the concentration of inhibitor that blocks 50% of radioligand binding. In a typical competition experiment, the binding of a fixed concentration of radioligand is inhibited by increasing concentration of an unlabeled ligand. The amount of radioligand that is bound to the receptor $[B]$ is,

$$[B] + [B_o] - f [B_o] / (1 + [I] / IC_{50}) \ldots \ldots \ldots (5)$$

Where, $[B_o]$ is the amount of radioligand in the absence of inhibitor and $f [B_o]$ is the amount of bound radioligand displaced by inhibitor.

The nonlinear regression analysis can be used to fit Eq. (5) to experimental data to provide an estimate of the $IC_{50}$ value.

$$IC_{50} = (1 + [L] / K_d) Ki \ldots \ldots \ldots (6)$$

The $IC_{50}$ value can also be calculated according to the Cheng-Prusoff equation (1973) as mentioned above.

### 5.3.3 Affinity of Various Thiazolidin-4-one Arecoline Derivatives towards M1 Receptor in Rat Cortex

Displacement of $[^3H]$ QNB binding to M1 receptor by various arecoline derivatives were studied in membranes obtained from cortex of control rats. The displacement assays were performed to calculate the $Ki$ and values of $IC_{50}$ for each arecoline derivative. The compounds used for the displacement studies were M1 receptor related compounds. All compounds which showed high affinity towards M1 receptor displaced the radioligand binding in a monophasic manner, suggesting the
inhibition of radioligand binding to a single class of receptors by various class of drugs (Table-1).

50 μg protein obtained from cortical membranes of rat brain were incubated with various different concentrations of arecoline derivatives (500 nM- 500 μM) and 0.16 nM of [3H] QNB, in final volume of 200 μL at 37°C for 2 hrs. At the end of incubation period, reaction was stopped by the addition of ice-cold phosphate assay buffer pH 7.4, and rapidly filtered through GF/B filters and washed twice with same ice-cold phosphate assay buffer pH 7.4. The filters were dried at 60°C and punched into the scintillation vials. Subsequently, 5 ml of scintillation cocktail was added and the radioactivity was counted after overnight equilibration (12-16 hrs) in a Packard liquid scintillation counter with an efficiency of 65%

5.3.4 Results and Discussion

Muscarinic receptors mediate a variety of physiological response to the neurotransmitter acetylcholine in central and peripheral nervous system. M1 receptor have been identified through molecular biological studies and characterized in rat brain tissue using pharmacological technique.

The affinity of the compounds for muscarinic receptor sites in the rat brain was determined by in vitro receptor binding studies. The ability of the compounds to displace tritiated [3H] QNB, a potent subtype nonselective muscarinic agonist, was interpreted as the affinity for the “agonist conformational sites” of the muscarinic receptor sites. Displacement of QNB, a selective antagonist for M1 muscarinic receptor was used to estimate the affinity for M1 receptor sites in hippocampus.

The efficacy of muscarinic agonists is generally measured by three biological methods: by comparing ratios of binding data, by testing the compounds in functional pharmacological models using isolated organs, which differ in receptor reserve for the various muscarinic receptor subtypes and by measuring effects on second messenger system.

The affinity of various potent thiazolidin-4-ones arecoline derivatives in the form of Ki and IC50 values are given in Table-1 and Fig-3.
The pharmacological studies are described in the experimental section. The IC$_{50}$ and Ki values for inhibition of binding tritiated muscarinic ligands by the compounds studied in this work are shown in Table-1. The efficacy of muscarinic agonist is generally measured by displacement methods by comparing ratio of binding data, by testing compounds in functional pharmacological models using isolated organs, which differ in receptor reserve for the M1 receptor and by measuring effects on second messenger system.

The affinity for the muscarinic agonist of thiazolidin-4-ones arecoline derivatives in rat brain cerebral cortex is as shown in Figure-3. These derivatives exhibited 50% activity of IC$_{50}$ values is 14b-810μM, 14e-275μM, 14f-250μM, 14j-100μM, 14k-284 μM and 14m- 363 μM, and Ki values is 14b-149μM, 14e-50μM, 14f-49μM, 14j-19μM, 14k-53μM and 14m-66μM (Table-1) respectively.

The muscarinic activity obtained for compounds 14b, 14e, 14f, 14j, 14k and 14m is conformed by the biochemical finding of the rat brain cerebral cortex. The most of the compounds screened did not show any agonistic activity. The results obtained from the synthesized compounds were compared with that of arecoline.

The compound 14m showed less activity to M1 receptor than 14j, which has high affinity than arecoline basic moiety. The presence of diphenylamine in 14j is probably responsible for extreme potent and selective agonistic activity. Similarly the change in substitution on the thiazolidin-4-ones ring in 14e, 14f, 14k, 14m and 14b have good affinity towards M1 receptor and has good agonistic activity. Compounds 14a and 14h with halogen atom and 14i with carboxyl group in aromatic ring attached to nitrogen atom of thiazolidin-4-ones has exhibited less affinity. In addition compounds 14c and 14g having less carbon atoms in alkyl chain attached to nitrogen atom of thiazolidin-4-ones has also exhibited less affinity.

Thiazolidin-4-one arecoline derivatives were tested their for M1 receptor binding affinity and agonistic activity. Receptor binding affinity for M1 receptor expressed in rat brain was assessed by radioligand binding assay using the labeled antagonist $[^3]$H QNB (quinuclidinyl benzilate). In the above set of derivative 14j has exhibited very high affinity towards M1 receptor with Ki value less than 19μM. When compared arecoline (Ki value 86μM). The compounds 14f, 14k, 14m and 14b also
very good affinity towards M1 receptor, i.e. Ki values 49μM, 53μM, 50μM, 66μM and 149μM respectively. (Table-1). Their increasing order of affinity is 14f > 14k > 14e > 14m > 14b (Figure 4).

If we compare the activity of the compounds with their structure i.e. structure activity relationship, we can deduce that, the compounds 14j, 14k and 14m having phenyl ring attached directly to the nitrogen of the thiazolidin-4-ones ring have shown very good affinity. In these compounds we observe that electron donating and withdrawing group at meta and para-position. Compound 14j increase the activity by donating electron to thiazolidin-4-ones ring. Where as electron withdrawing group at para position 14k decrease the activity of the compounds by withdrawing electrons from thiazolidin-4-ones ring. Where as OH group at meta position of ring have remarkable influence of on the activity of compounds (14m).

Comparison between 14a and 14f shows that, these compounds are having electron donating alkyl groups at nitrogen of the thiazolidin-4-ones. In these compounds the presence of long chain of carbon atoms has decreases the affinity compared to compounds 14h, 14j, 14k and 14m (Figure 4).

The aim of our research is to identify M1 selective muscarinic agonists capable of crossing the blood brain barrier (BBB). The compound 14j (R=4-aminodiphenylamine) displayed very high degree of M1 receptor selectivity with the Ki =19μM and IC 50=100 μM. This suggests that BBB penetration of the compound 14j is good, when compared to the arecoline which has the Ki of 86μM. The compound 14e, 14f and 14k shows the Ki value of 49μM, 50μM and 53μM respectively. These compounds showed less affinity to M1 receptor than 14j which has high affinity than the arecoline basic moiety.
Table 1: Affinity of Various Thiazolidin-4-one Arecoline Derivatives to M1 Receptor in Rat Cortex.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ki μM</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
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<tbody>
<tr>
<td>14a</td>
<td>-</td>
<td>-</td>
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<tr>
<td>14b</td>
<td>149</td>
<td>810</td>
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<tr>
<td>14c</td>
<td>-</td>
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<td>14d</td>
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<tr>
<td>14e</td>
<td>50</td>
<td>275</td>
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<td>14f</td>
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<tr>
<td>14j</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>14k</td>
<td>53</td>
<td>284</td>
</tr>
<tr>
<td>14l</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14m</td>
<td>66</td>
<td>363</td>
</tr>
<tr>
<td>Arecoline</td>
<td>86</td>
<td>469</td>
</tr>
</tbody>
</table>
Figure 2: Binding of $[^{3}\text{H}]$ QNB to Total M1 Receptor in Membrane Obtained from Cortex. Non Specific Binding was Defined by Using (100 nM). Saturation for $[^{3}\text{H}]$ QNB Binding to Cortical Total M1 Receptor were Shown in the Inset.
Figure 3: Displacement of $[^3H]$ QNB Binding to M1 Muscarinic Receptor by Two Potent Thiazolidine-4-one Arecoline Derivatives 14f and 14j in Rat Cortex.
Figure 4: The Most Significant Molecules Affinity on M1 Receptor.
Definition of Some Important Terms

**Affinity**: a measure of how tightly a drug binds to a receptor mathematically, affinity is $1/K_d$ that is the higher the affinity lower the $K_d$ and tighter the binding of ligand to the receptor or in other words the higher the affinity the is lower dissociates from the receptor.

**Bmax**: it shows the density of the receptor site in a particular preparation. It is usually determined in a saturation experiment as the maximum number of receptor occupied at saturating concentration of radioligand.

The unit of Bmax is mol/g of tissue / protein.

**$K_d$**: the dissociation equilibrium constant of a drug for a receptor, $K_d$ is a measure of the affinity of a drug for a receptor. The lower the $K_d$ the tighter the drug binds to the receptor and the higher the affinity of the drug for the receptor.

**$K_i$**: The equilibrium dissociation, constant for a competitive inhibitor of the receptor.

Note: The competitive inhibitor can be an agonist or an antagonist it is called competitive inhibitor because its value is determined by measuring the ability of the unlabelled drug to compete with a radioligand drug for the receptor.

**IC$_{50}$**: IC$_{50}$ is the concentration of the inhibitor required to inhibit the binding of a radioligand by 50%.
5.4 BEHAVIORAL TESTING OF THIAZOLIDIN-4-ONE ARECOLINE DERIVATIVES

5.4.1 Introduction to Elevated Plus Maze

The elevated plus maze (EPM) served as the exteroceptive behavioral model (wherein the stimulus existed outside the body) to evaluate learning and memory in rat. The apparatus consisted of two open arms (16 cm x 5 cm) and two covered arms (16 cm x 5 cm x 12 cm). The arms extended from a central platform (5 cm x 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day, each rat was placed at the end of an open arm, facing away from the central platform (Figure 5). Transfer latency (TL) was taken as the time taken by the rat to move into any one of the covered arms with all its four legs. TL was recorded on the first day. If the rat did not enter into one of the covered arms within 90 s, it was gently pushed into one of the two covered arms and the TL was assigned as 90 s. The rat was allowed to explore the maze for 10 s and then was returned to its home cage. Memory retention was examined 24 h after the first day trial on the second day\textsuperscript{16-17}.

![Figure 5: Elevated Plus Maze](image-url)
5.4.2 Transfer Latency (TL) on Elevated Plus Maze (EPM)

EPM is a tool to assess learning and memory performance in laboratory animals. It has also been extended to measure the cognitive performance, especially the spatial long-term memory in rats and mice. The elevated plus maze, which was introduced by Pellow (1985) with rats and by Lister (1987) with rats consists of two open and two enclosed arms, and is based on the apparent natural aversion of rodents to open and high spaces and is used to measure the anxiety state in animals. Animals spend more time in the enclosed arms than the open arms, because they dislike the open arms. The aversive quality of the open arms is not apparent until the animals enter them. Based on the parameter, Itokh et al. have demonstrated that transfer latency (the time in which the animal moves from the open arm to closed arm) was markedly shortened if the animal had previously experienced entering the open arms, and this shortened transfer latency (TL) has been shown to be related to memory process. Accordingly, the TL from the second day onwards was shorter than on the first day of exposure of animals to the plus maze.

Several existing methods for evaluation of learning and memory in animals are based on positive or negative reinforces such as food or electric shocks, although, this method does not use reinforces and can be used as model for memory and learning.

This test relies on the inherent conflict between exploration of a novel area and avoidances, of its aversive features.

Learning and memory were previously evaluated by using elevated plus maze test in rats. We have used this method to test antiamnesic property of newly synthesized arecoline derivatives for reversing scopolamine induced memory loss (as short term preclinical model for dementia). Scopolamine is muscarinic receptor antagonist causes memory impairment and arecoline derivatives are probable muscarinic agonist, enhance memory and cognition.

In the present study experimental animals are grouped in to six, one group serve as control rats (vehicle treated), second group as scopolamine treated rats (dementia model) and third group of rats (scopolamine + arecoline derivatives 1). Fourth group of rats(scopolamine + arecoline derivatives 2), fifth group of rats(arecoline derivatives 1)
and sixth group of rats(arecoline derivatives 2). All the six groups were used to measure TL on first day (test acquisition or learning) and TL on second day (test retrieval or memory) statistical analysis was done to evaluate the efficacy of these derivatives.

This decrease in TL was observed when rat spent even 10 sec in enclosed arm after entering it.

The plus maze apparatus for rats consist of two open arms with a dimension of 50 x 20 cm and two closed arms with a dimension of 50 x 10 x 40 cms facing each other with an open roof. A fine white line may be drawn in the middle of the floor of each enclosed arm. The entire maze is elevated at 50 cm.

The maze may be made of wood, metal or Plexiglas, in the present experiment we have used wooden plus maze. EPM was designed to provide measure of anxiety that were relatively uncontaminated by changes in overall motor activity and has been extensively validated by pellow et al (1985) using behavior, physiological and pharmacological measures.

Muscarinic agonist and antagonist impair locomotor function connected to cholinergic function and also impair spatial memory in rats.

5.4.3 Materials and Methods

Materials

1. Male wistars rats  200 – 250 gm
2. Cages
3. Elevated plus maze
4. Drugs (Arecoline derivatives)
5. Needles, syringes.
6. Scopolamine
7. Alcohol
8. Cotton
9. Glouse
10. Stop watch
11. Torch
12. Standard volumetric flask (10, 25 ml)
13. Distilled water
14. Animal weighing balance
15. Mascara.

Methods

1. Elevated plus maze (EPM) is experiment is carried out in a dark semi sound proof room with a zero candle red light placed at the center position of tarce in room.

2. Wooden plus maze was placed on the ground at the center of the room.

3. Male wistar rats aged 3 months and weighing 200-250 grams are selected for the experiment

4. They were grouped into six, each group consist of 8 animals, groups were named as
   a) Control (Vehicle treated)
   b) Scopolamine treated
   c) Test Drugs 1st day treated (Thiazolidin-4-one arecoline derivatives, 14a-m)
   d) Test Drugs 2nd day treated (Thiazolidin-4-one arecoline derivatives, 14a-m)
   e) Scopolamine + Test Drug 1st day treated (Thiazolidin-4-one arecoline derivatives, 14a-m)
f) Scopolamine + Test Drug 2nd day treated (Thiazolidin-4-one arecoline derivatives, \textbf{14a-m})

Six animals are placed in each cage and 8 cages are used for the experiment. In order to differentiate animals in each cage mascara colour marking are used on different parts of the rat. On head (H), on body (B), on tail (T), without colour (C), on head body (HB) and on head tail (HT).

5. Each batch of experiment involved two days and in which two test derivatives were evaluated.

6. Before the commencement of experiment all the cages are placed in light room

7. 20 min before the drug administration to each cages animal are brought into dark room for acclimatization.

8. After 20 min each animal in the cage was treated with vehicle (- mg/kg body weight)

   a) Scopolamine (- mg/kg body weight)

   b) Scopolamine + Test Drug I (- mg/kg body weight)

   c) Scopolamine + Test Drug II (- mg/kg body weight)

   d) Test Drug I (- mg/kg body weight)

   e) Test Drug II (- mg/kg body weight)

Injections were given to each animal by intraperitonial (IP) route at the interwell of 3 mins.

9. Exactly after 30 mins of injection each animal was placed on the extreme end of open arm of EPM and allowed the fresh rat (Previously not used to explore plus maze) to explore EPM.

10. Allowed the animal to explore EPM for 90 seconds Transfer latency for first day was (TL1) measured by noting the time on stop - watch for the animal to reach extreme end of the open arm to closed arm. (All the four legs should be
inside the closed arm). And allowed animal to stay for 10 sec in the closed arm for acquisition (learning) purpose. In case, rat fails to move from open arm to closed arm within 90 sec. Push the rat from open arm to closed arm and allowed the animal to stay for 10 sec. in closed arm.

11. From the closed arm after 10 sec. Animal is placed back to the original cage.

12. After subjecting each animal to explore EPM, all the four arms of EPM were cleaned with cotton soaked in alcohol, before subjecting next animal to explore EPM.

13. In the similar way remaining animals in other cages were allowed to explore EPM.

14. After 24 hours, the experiment was repeated with the similar treatments to same animals and similar exposure to EPM to get second day transfer latency (TL2)

15. Difference between TL2 and TL1 is used for statistical analysis to evaluate the efficacy of test derivatives for learning and memory.
Table 2: Study of Antiamnesic Effect of Thiazolidin-4-one Arecoline Derivatives (14a-m) against Scopolamine Induced Memory Loss in 1st Day.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment Group</th>
<th>Treatment (dose) (mg/kg,ip)</th>
<th>Transfer Latency (TL)</th>
<th>Average Difference Transfer Latency(TL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TL1</td>
<td>TL2</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>-</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Scopolamine</td>
<td>0.4</td>
<td>62</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>14a</td>
<td>0.1</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>14b</td>
<td>0.1</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>14c</td>
<td>0.1</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>14d</td>
<td>0.1</td>
<td>46</td>
<td>42</td>
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<tr>
<td>7</td>
<td>14e</td>
<td>0.1</td>
<td>34</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>14f</td>
<td>0.1</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>14g</td>
<td>0.1</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>10</td>
<td>14h</td>
<td>0.1</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>11</td>
<td>14i</td>
<td>0.1</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td>12</td>
<td>14j</td>
<td>0.1</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td>14k</td>
<td>0.1</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>14</td>
<td>14l</td>
<td>0.1</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>15</td>
<td>14m</td>
<td>0.1</td>
<td>18</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 6: Antidementia Activity of Thiazolidin-4-one Arecoline Derivatives (14a-m) 1st Day.
Table 3: Study of Antiamnesic Effect of Thiazolidin-4-one Arecoline Derivatives + Scopolamine (14a-m+Scopolamine) against Scopolamine Induced Memory Loss in 1st Day.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment Group</th>
<th>Treatment (dose) (mg/kg,ip)</th>
<th>Transfer Latency (TL)</th>
<th>Average Difference Transfer Latency(TL)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
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<td>11</td>
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<tr>
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<td>59</td>
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<tr>
<td>3</td>
<td>14a + Scopolamine</td>
<td>0.05+0.05</td>
<td>48 TL1, 44 TL2</td>
<td>47</td>
</tr>
<tr>
<td>4</td>
<td>14b+ Scopolamine</td>
<td>0.05+0.05</td>
<td>26 TL1, 22 TL2</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>14c+ Scopolamine</td>
<td>0.05+0.05</td>
<td>50 TL1, 40 TL2</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>14d+ Scopolamine</td>
<td>0.05+0.05</td>
<td>48 TL1, 42 TL2</td>
<td>45</td>
</tr>
<tr>
<td>7</td>
<td>14e+ Scopolamine</td>
<td>0.05+0.05</td>
<td>25 TL1, 19 TL2</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>14f+ Scopolamine</td>
<td>0.05+0.05</td>
<td>26 TL1, 16 TL2</td>
<td>21</td>
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<tr>
<td>9</td>
<td>14g+ Scopolamine</td>
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<td>42 TL1, 36 TL2</td>
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<tr>
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<td>14h+ Scopolamine</td>
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<td>46 TL1, 30 TL2</td>
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<tr>
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</tr>
<tr>
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<td>14j+ Scopolamine</td>
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<td>22 TL1, 18 TL2</td>
<td>20</td>
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<tr>
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<td>0.05+0.05</td>
<td>28 TL1, 16 TL2</td>
<td>23</td>
</tr>
<tr>
<td>14</td>
<td>14l+ Scopolamine</td>
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<td>45 TL1, 33 TL2</td>
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<tr>
<td>15</td>
<td>14m+ Scopolamine</td>
<td>0.05+0.05</td>
<td>30 TL1, 30 TL2</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 7: Antidementia Activity of Thiazolidin-4-one Arecoline Derivatives + Scopolamine (14a-m+Scopolamine) 1st Day.

Sco = Scopolamine
Table 4: Study of Antiamnesic Effect of Thiazolidin-4-one Arecoline Derivatives (14a-m) against Scopolamine Induced Memory Loss in 2\textsuperscript{nd} Day.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment Group</th>
<th>Treatment (dose) (mg/kg/ip)</th>
<th>Transfer Latency (TL)</th>
<th>Average Difference Transfer Latency(TL)</th>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
<td>Scopolamine</td>
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<td>55 53 54</td>
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<tr>
<td>3</td>
<td>14a</td>
<td>0.1</td>
<td>44 38 42</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14b</td>
<td>0.1</td>
<td>28 16 22</td>
<td></td>
</tr>
<tr>
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<td>14c</td>
<td>0.1</td>
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<td></td>
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<tr>
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<td>14d</td>
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<td>22 18 20</td>
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<td>14f</td>
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<td>20 16 18</td>
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</tr>
<tr>
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<td>14g</td>
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<td>14h</td>
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</tr>
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<td>14j</td>
<td>0.1</td>
<td>18 14 16</td>
<td></td>
</tr>
<tr>
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<td>14k</td>
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<td>22 16 19</td>
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<td>14</td>
<td>14l</td>
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</tr>
<tr>
<td>15</td>
<td>14m</td>
<td>0.1</td>
<td>26 20 23</td>
<td></td>
</tr>
</tbody>
</table>
Figure-8: Antidementia Activity of Thiazolidin-4-one Arecoline Derivatives (14a-m) 2\textsuperscript{nd} Day.

![Graph showing antidementia activity of thiazolidin-4-one arecoline derivatives (14a-m) on 2\textsuperscript{nd} day. The graph compares transfer latency (seconds) for different groups including control, scopolamine, and various thiazolidin-4-one arecoline derivatives.]
Table 5: Study of Antiamnesic Effect of Thiazolidin-4-one Arecoline Derivatives + Scopolamine (14a-m + Scopolamine) against Scopolamine Induced Memory Loss in 2\textsuperscript{nd} Day.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Treatment Group</th>
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<td>TL2</td>
</tr>
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<td>8</td>
</tr>
<tr>
<td>2</td>
<td>Scopolamine</td>
<td>0.4</td>
<td>55</td>
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<td>14k+ Scopolamine</td>
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<td>14</td>
<td>14l+ Scopolamine</td>
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<tr>
<td>15</td>
<td>14m+ Scopolamine</td>
<td>0.05+0.05</td>
<td>34</td>
<td>20</td>
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</table>
Figure 9: Antidementia Activity of Thiazolidin-4-one Arecoline Derivatives + Scopolamine (14a-m+Scopolamine) 2nd Day.
5.5 CONCLUSION

From the structure activity relationship, it is observed that the thiazolidin-4-ones ring containing single or non fused heterocyclic moieties (14j and 14f) showed a better muscarinic agonistic activity, than fused heterocyclic moieties (14k, 14e, 14m and 14b), which was also observed from reversing amnesic effect of scopolamine induced memory loss in elevated plus maze in rats. The data shown in Tables 2-5 and Figures 6-9 will support the conclusion drawn.
REFERENCES