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# Muscarinic receptor 1 agonist activity of novel *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives in Alzheimer's presenile dementia models

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ABSTRACT

Earlier we have reported the effect of arecoline thiazolidinone and morpholino arecoline derivatives as muscarinic receptor 1 agonists in Alzheimer's presenile dementia models. To elucidate further our Structure–Activity Relationship (SAR) studies on the chemistry and muscarinic receptor 1 binding efficacy, a series of novel carboxamide derivatives of 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)morpholine molecule have been designed and synthesized as a new class of M1 receptor agonists with a low toxicity effect profile that enhances memory function in animal models of Alzheimer's presenile dementia and also modulates the APP secretion from rat brain cerebrocortical slices by activating M1 receptor in vitro. Results suggest that compound **9b** having methyl group at the *para* position of the aryl group attached to the carboxamide of morpholino arecoline could emerge as a potent molecule having antidementia activity.



## 1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease that involves a gradual and progressive memory loss and dementia.[1] Neuropathologically, the disease is characterized by the presence of extracellular deposits of amyloid β-peptide (Aβ) as fibril aggregates that form senile plaques, intracellular neurofibrillary tangles and degeneration of presynaptic cholinergic neurons that ascend from the basal forebrain to cortical and hippocampal areas.[2,3] The M1 acetylcholine muscarinic receptor (mAChR) is expressed in the cerebral cortex and hippocampus and its major role is in cognitive processing including short-term memory;[4,5] however, muscarinic agonists were not effective in AD treatment, inter alia, due to the lack of selectivity for the M1 mAChR, narrow safety margin, very low bioavailability and poor tolerability.[6,7]

Activation of M1 mAChR by agonist modulates not only learning and memory;[4,8] but also protects cells from apoptotic effects induced by $H_2O_2$ including DNA damage, oxidative stress and mitochondrial impairment in cortical neurons.[9] M1 agonist also blocks caspase activation in neurons deprived of serum;[10] increases the non-amyloidogenic processing of the β-amyloid precursor protein (APP), a membrane associated glycoprotein containing within its sequence the amyloid β-protein (Aβ) which is the major component of the senile plaques found in brains of Alzheimer's patients;[11,12] reduces the Aβ-peptide production;[13–15] reduces the tau hyperphosphorylation;[16,17] increases inhibitory postsynaptic currents,[18,19] etc. However, the mechanism by which mAChR activation protects neurons from insults is not well understood.

In this study we have derivatized Arecoline molecule to develop new class of M1 receptor selective agonists for the possible treatment of AD dementia. Arecoline, an alkaloid obtained from the betel nut (*Areca catechu*), the fruit of a palm tree, has been used previously as centrally active muscarinic agents.[20] The lack of M1 selectivity and efficacy due to dose limiting side effects associated with M2 and M3 muscarinic receptors subtype stimulation has produced disappointing results.[20] Replacement of the ester functionality of arecoline with either the 3-alkoxy-1,2,5 thiadiazole[21] or the 3-alkyl-1,2,4-oxadiazole,[22] has produced very potent muscarinic agonists. However, the systematic removal of a heteroatom in the 3-methyl-1,2,4-oxadiazole giving oxazoles and furans caused a decrease in affinity for the agonist binding site. The two isomers, 2-methyl-1,2,4-oxadiazole and 5-methyl-1,2,4-oxadiazole,[22] also had shown lower affinities for M1 muscarinic receptor [see the structure of Arecoline and its above mentioned derivatives, Fig. 1].

Previously we have reported, muscarinic M1 subtype selectivity for arecoline thiazolidinone[23] and for 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)morpholine.[24,25] In a continued effort to discover

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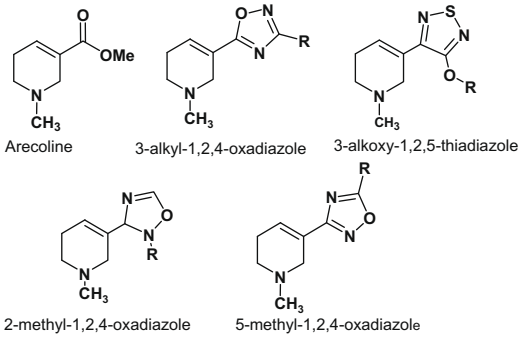



**Figure 1.** Arecoline and arecoline derivatives.

**Table 1**
In vitro affinity of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**) towards M1 receptor of male Wistar rat cortex synaptosomal membrane

| Compounds | $K_i$ (μM) | $IC_{50}$ (μM) |
|---|---|---|
| **9a** | 19 ± 3.22 | 76 ± 5.51 |
| **9b** | **0.42 ± 0.06** | **1.6 ± 0.13** |
| **9c** | 1.3 ± 0.08 | 4 ± 1.50 |
| **9d** | Nil | Nil |
| **9e** | Nil | Nil |
| **9f** | 126 ± 13.83 | 618 ± 20.35 |
| **9g** | 48 ± 7.32 | 142 ± 11.14 |
| **9h** | 65 ± 6.12 | 215 ± 13.50 |
| Arecoline | 88 ± 11.25 | 460 ± 18.18 |

less toxic arecoline class of muscarinic agonists and to further improve their selectivity and potency, here, we are reporting our findings of *N*-aryl carboxamide substituted 3-morpholino arecoline **9**(**a–h**) derivatives (Scheme 1) along with their in vitro muscarinic receptor 1 binding studies by using [$^3$H]QNB with male Wistar rat brain synaptosomal membrane; estimation of second messenger Inositol (1,4,5)-trisphosphate (IP3) formation; and modulation of APP secretion in rat brain cerebrocortical slices in response to M1 receptor activation by synthesized molecules, and also in vivo evaluation of memory and learning in male Wistar rats (passive avoidance tasks, plus and Y maze studies), as another potent M1 receptor agonist for the symptomatic treatment of Alzheimer's dementia.

## 2. Results and discussion

Structure–Activity Relationship (SAR) can be drawn from the in vitro radioligand affinity assay, which was carried out to find out the affinity of the synthesized *N*-aryl carboxamide substituted 3-morpholino arecoline **9**(**a–h**) derivatives for the cortical M1 receptor of rat brain (Scheme 1, Table 1 and Fig. 2). The most potent compound among all tested derivatives, **9b** ($K_i$ = 0.42 μM), is one with methyl group at the *para* position of the aryl group attached to the carboxamide of morpholino arecoline. Substitution of chlorine group (**9c**), at *para* position also showed good affinity towards the M1 receptor in vitro ($K_i$ = 1.3 μM). However, disubstituted chlorine (**9e**) at 2 and 5 positions and also tertiary butyl substituted derivative (**9d**) at the *para* position of the aryl group attached to carboxamide of morpholino arecolines, showed no affinity for the M1 receptor in vitro.

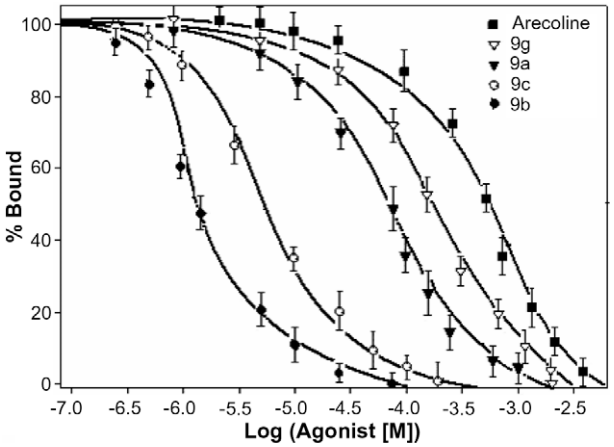
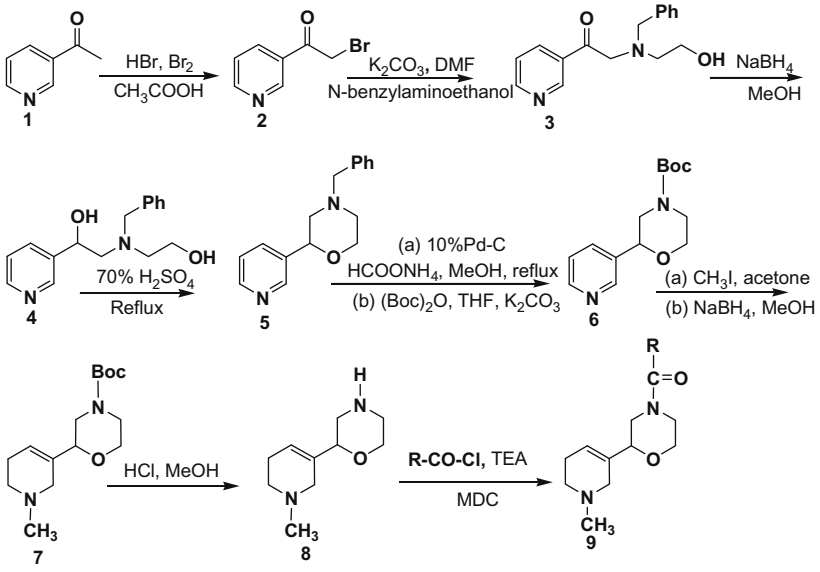



**Figure 2.** Displacement graphs of four most potent compounds **9b**, **9c**, **9a** and **9g**. The displacement studies were done with 0.2 nM [$^3$H]QNB and different concentration of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**). The mean values of % bound are plotted against log of displacer concentration. $IC_{50}$ and $K_i$ values are obtained from LIGAND–DRUG programme.




**Scheme 1.**

On the other hand substitution of nitro group ($NO_2$) at the different positions of the aryl group attached to the carboxamide of morpholino arecolines **9**(**f–h**), reduced the affinity and potency of the compounds for the M1 receptor. Among the derivatives having electron withdrawing $NO_2$ group at the aryl group, $NO_2$ at *meta* position **9g** ($K_i$ = 48 μM), showed considerable high affinity when compared it at *para* position **9h** ($K_i$ = 65 μM), while $NO_2$ at *ortho* position **9f** ($K_i$ = 126 μM) showed least affinity. Compound **9a** having phenyl group without any substitution on it, attached to the carboxamide of morpholino arecoline showed moderate affinity ($K_i$ = 19 μM) to the muscarinic receptor 1 in vitro.

Muscarinic acetylcholine receptors (mAChR) play an important role, among several events, in β-amyloid precursor protein (APP) processing, as it was demonstrated in various cell lines transfected with M1 and M3 receptors.[26,27,15] Elevation of cytoplasmic calcium and activation of protein kinase C, in these cells, play a role in muscarinic-mediated APP secretion,[28] and it was also demonstrated that phospholipase C (PLC), which activates phosphoinositide (PI) hydrolysis, is involved in transduction signaling.[29,30] Therefore, we planned to measure the efficacy and potency of our synthesized compounds **9a**, **9b**, **9c** and **9g**, which had shown high affinity for the M1 receptor in radioligand binding studies, to elevate the IP3 formation in rat cerebrocortical slices and also to examine the modulation of APP secretion by activating M1 receptor with these compounds in rat brain cerebrocortical slices. Basal IP3 levels were found to be 0.64 pmol/mg proteins, and it was significantly increased by activating the M1 receptor with the compound **9b**. Compounds **9c** and **9a** could also elevate the IP3 formation when it was compared with basal IP3 levels, but values were not significant (Table 2). The potency of these compounds to elevate the basal IP3 levels was compared with IP3 levels elevated by acetylcholine iodide, which represents M1 receptor-linked PLC activity (Fig. 3).

In vitro modulation of APP secretion in rat brain cerebrocortical slices by activating M1 receptor with our synthesized M1 receptor agonists were studied. Secreted APP from cerebrocortical slices appeared as two protein bands with an apparent molecular weight of 105,000 (major band) and 82,000 (minor band). These APP bands may correspond to secreted forms of $APP_{751}$ and $APP_{695}$.[11] Alternatively, they may correspond to mature and immature (non-glycosylated) forms and activation of M1 receptor by our synthesized molecules evoked APP release in rat brain slices. We used novel compounds **9a**, **9b**, **9c** and **9g**, to study their efficacy and potency to modulate the APP secretion by activating M1 receptor, as they already had shown high affinity for M1 receptor in binding studies and also have elevated IP3. The secretion of APP from the rat cerebrocortical slices was significantly increased by molecule **9b** (Fig. 4), whereas **9a**, **9c** and **9g** were not very effective in elevating the APP levels. The induction of APP released by **9b** was inhibited by atropine, indicating that the effect is mediated via the activation of mAChRs. These results show similarity to the neural cells that express M1 and M3 receptors which also respond to muscarinic activation by increasing APP secretion.

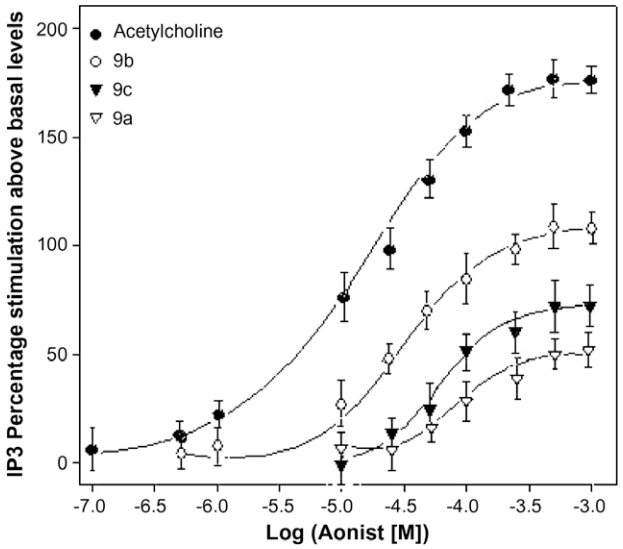



**Figure 3.** Inositol (1,4,5)-trisphosphate (IP3) levels were estimated using [$^3$H]myo-inositol, in rat brain cerebrocortical slices after stimulating the M1 receptor by different concentration of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a**–**h**) and significance was compared with the basal IP3 level, and their potency to elevate the IP3 formation were determined by comparing it with IP3 levels elevated by acetylcholine iodide as described in experimental section. This figure represent scatchard graph of three most potent compounds (**9b**, **9c** and **9a**) among all the tested derivatives **9**(**a**–**h**). Data points are the means of three experiments, each assayed in duplicate.

**Table 2**
In vitro effect of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a**–**h**) on the formation of Inositol (1,4,5)-trisphosphate (IP3) levels in the cerebrocortical slices of the rat brain

| Agonists stimulation of M1 receptor (100 μM) | IP3 Concentration (pmol/mg of protein) |
|---|---|
| Control (Basal) | 0.64 ± 0.12 |
| **9a**-Stimulated | 0.82 ± 0.25 |
| **9b**-Stimulated | **1.19 ± 0.33**$^*$ |
| **9c**-Stimulated | 0.94 ± 0.31 |
| **9d**-Stimulated | 0.63 ± 0.17 |
| **9e**-Stimulated | 0.64 ± 0.30 |
| **9f**-Stimulated | 0.65 ± 0.17 |
| **9g**-Stimulated | 0.80 ± 0.21 |
| **9h**-Stimulated | 0.64 ± 0.20 |
| Acetylcholine-stimulated | 1.60 ± 0.09$^*$ |

The efficacy of novel *N*-aryl carboxamide substituted 3-morpholino arecoline **9**(**a**–**h**) derivatives (100 μmol/l) for the M1 receptor was measured by their potency to elevate the basal IP3 levels, and it was compared with IP3 levels elevated by acetylcholine iodide (20 μmol/l), which represent M1 receptor-linked PLC activity. The values are mean and standard deviation of three experiments, each assayed in duplicate. IP3 formation was expressed as picomoles of [$^3$H]IP3 formed per milligram of tissue. $n$ = 6; $^*p < 0.05$.

The foresaid in vitro M1 receptor binding studies and subsequently IP3 formation studies and modulation of APP secretion by activating M1 receptor, formed a basis for extending correlation further to in vivo pharmacological studies to ascertain applicability of the synthesized molecules **9**(**a–h**) in scopolamine (muscarinic antagonist) induced dementia models, using memory and learning experiments (passive avoidance tasks, plus and Y maze studies), as it has been demonstrated previously that cholinergic transmission appears to be essential for learning and memory process.[31,32] In accordance with the degree of affinity and potency of synthesized compounds **9**(**a–h**) in vitro binding experiments, elicited almost anticipated level of pharmacological actions in reversing scopolamine induced dementia in vivo (Table 3 and Fig. 5). Two synthesized morpholino arecoline derivatives **9b** and **9c** are best among all the derivatives **9**(**a–h**) in reversing scopolamine-induced dementia by making rats to commit less number of mistakes (No. of mistakes done 11 and 15, respectively), when compared it with the number of mistakes done by control rats (6 mistakes) and scopolamine-treated group (30 mistakes). Compound **9g** also significantly reversed the scopolamine induced memory loss. Other compounds showed least or no activity against scopolamine induced memory loss in rats (Table 3).

The plus maze experiments for synthesized derivatives **9**(**a–h**) measures the transfer latency (TL) in seconds taken to reach from one extreme end of open arm to one of the closed arms in plus maze. Difference in TL in seconds between 1st day and 2nd day

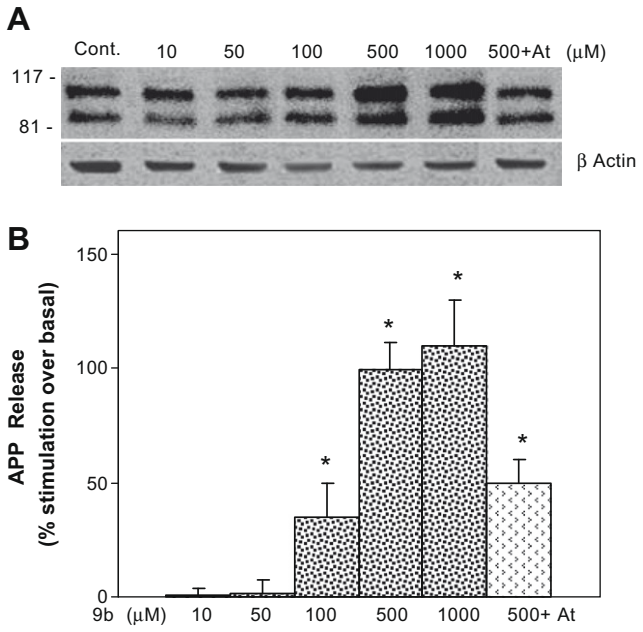



**Figure 4.** (A) M1 mAChR activation by **9b** induces APP secretion from rat cerebrocortical slices. Protein extracts from rat brain slices stimulated with different concentration of **9b**, were prepared and electrophoresed on a PAGE gel. Following western blotting, immunodetection with specific antibodies was carried out to detect the levels of secreted APP on the blots. The last lane demonstrates the blockade of **9b** (500 μM) induced APP secretion by 10 μM atropine (At). (B) The intensity of bands, specific for the secreted APP protein was measured densitometrically. The values were graphed as mean and standard error values of six experiments each performed in duplicates for the **9b** treated rat brain slices, and it was compared to not treated control rat brain cerebrocortical slices in terms of fold increase or decrease. *Indicates measurements significantly different to non-treated control APP levels (*$p < 0.05$).

for scopolamine-treated group, and test compound along with scopolamine-treated groups were compared to evaluate learning (TL1) and memory (TL2). Derivatives **9b** and **9c** significantly reversed acute memory loss and learning impairment in male Wistar rats (Table 4 and Fig. 6). Compound **9b** produced lesser TL for 1st day (TL1 = 33 s) compared to scopolamine-treated group (TL1 = 66 s), but on 2nd day (TL2 = 13 s) TL was lesser than 1st day (**9b**) indicating how it is helpful in reversing learning impairment as compared to rest of the derivatives. Overall difference between TL1 and TL2 were same for **9b**, **9c** (20 s), whereas for the control group it was 22 s. In contrary this, **9a** and **9g** molecules produced longer 1st day TL (TL1 = 53 s and 64 s, respectively) and 2nd day TL (TL2 = 35 s and 47 s, respectively) overall difference differs (TL1–TL2) were 18 s and 17 s, respectively, implying that these derivatives failed to reverse acute memory and learning impairment in male Wistar rats. TL for remaining compounds are given in Table 4. Similar results were obtained from Y maze experiment (data not shown).

The finding of this study is somewhat similar to our previous finding where arylthioureas are attached to the morpholino arecoline.[25] In both the studies, it is the position of the substitutes on the aryl group which determines the affinity and potency of the molecules for the M1 receptor. Substitution on the *para* position of the aryl group attached to either amide or thiourea of morpholino arecoline gives better affinity for the M1 receptor, when compared it at *meta* or at *ortho* positions. Electron donating group (e.g., methyl, **9b**) at the *para* position of aryl group attached to the carboxamide of morpholino arecoline has shown more affinity when compared with the electron withdrawing group (e.g., chloro, **9c**) at the *para* position. In contrary to this, in *N*-arylthioureas substituted morpholino arecoline derivatives electron withdrawing group (e.g., chloro) at the *para* position has shown better affinity when compared it with electron donating group (e.g., methoxy) at the *para* position.[25] However when aryl group is replaced with either alkyl group or with amino acids, it decreases the affinity and potency of the molecules for the M1 receptor (ongoing work in our laboratory).

## 3. Conclusion

In light of these findings, in vitro radioligand competitive binding assay for the M1 receptor; stimulation of IP3 formation by activating M1 receptor; modulation of APP secretion in rat brain cerebrocortical slices and in vivo pharmacological experiments for the synthesized compounds *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**), to test the relation between affinity of these molecules to M1 receptor and their potency to modulate the APP secretion and also their ability to reverse scopolamine-induced memory loss and learning impairment, for ascertaining their applicability in Alzheimer's dementia. We can conclude that position of substituents on the aryl group attached to the carboxamide of morpholino arecoline derivatives determines the affinity of the compounds for the M1 receptor. Substitution at the *para* position increases the affinity and potency of the molecules for the M1 receptor. The compound **9a** which dose not having any substituted group on the aryl group is found to have moderate affinity for the M1 receptor. But, when either methyl (**9b**) or chlorine (**9c**) group is introduced at the *para* position, it increases the affinity of the compounds several folds for the M1 receptor in vitro and showed greater potency in elevating the IP3 levels in rat cerebrocortical slices, and also have shown useful

**Table 3**
Study of antiamnesic effect of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**) against scopolamine induced memory loss

| S. No. | Experimental groups | Treatment (dose) mg/kg ip | Basal latency (s) of rat to reach shock free zone | | | Memory parameters | |
|---|---|---|---|---|---|---|---|
| | | | I | II | III | Latency (s) | Mistakes |
| 1 | Control group* | Saline (0.9%) | 18 | 9 | 5 | 2 | 6 ± 2 |
| 2 | Scopolamine treated group | 0.5 | 38 | 12 | 10 | 7 | 30 ± 9 |
| 3 | Scop. + **9a** | 0.5 + 0.5 | 29 | 12 | 10 | 6 | 25 ± 7 |
| 4 | Scop. + **9b*** | 0.5 + 0.5 | 23 | 10 | 8 | 4 | 11 ± 6 |
| 5 | Scop. + **9c*** | 0.5 + 0.5 | 22 | 11 | 7 | 3 | 15 ± 6 |
| 6 | Scop. + **9d** | 0.5 + 0.5 | 40 | 15 | 11 | 6 | 30 ± 10 |
| 7 | Scop. + **9e** | 0.5 + 0.5 | 38 | 13 | 11 | 9 | 31 ± 8 |
| 8 | Scop. + **9f** | 0.5 + 0.5 | 35 | 12 | 10 | 5 | 30 ± 9 |
| 9 | Scop. + **9g*** | 0.5 + 0.5 | 28 | 13 | 10 | 4 | 20 ± 7 |
| 10 | Scop. + **9h** | 0.5 + 0.5 | 35 | 15 | 10 | 5 | 28 ± 8 |
| 11 | Scop. + Arecoline | 0.5 + 0.5 | 30 | 13 | 10 | 5 | 23 ± 6 |

Results are expressed as mean (±SEM), $n = 10$, *$P < 0.05$. (Scop. = Scopolamine).

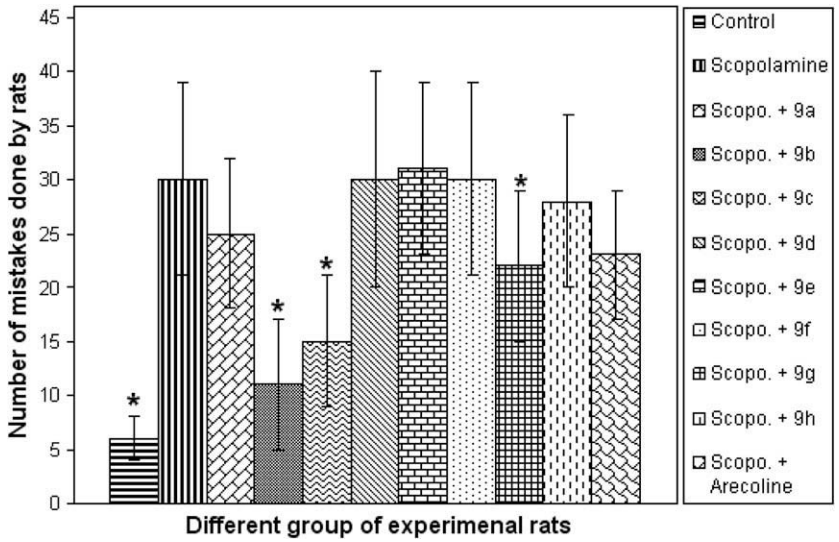



**Figure 5.** Antiamnesic effect of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a**–**h**) against scopolamine induced memory loss in male Wistar rats ($^{*}p < 0.05$), mean (±SEM).

**Table 4**
Acute studies of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a**–**h**) on learning and memory against scopolamine-induced memory loss and learning impairment on elevated plus maze in male Wistar rats

| S. No. | Experimental groups | Treatment (dose) (mg/kg, ip) | First day transfer latency (TL1) in s | Second day transfer latency (TL2) in s | TL1–TL2 |
|---|---|---|---|---|---|
| 1 | Control group* | Saline (0.9%) | 31 | 9 | 22 ± 8.5 |
| 2 | Scopolamine treated group | 0.5 | 66 | 53 | 13 ± 5.6 |
| 3 | Scop. + **9a** | 0.5 + 0.5 | 53 | 35 | 18 ± 6 |
| 4 | Scop. + **9b*** | 0.5 + 0.5 | 33 | 13 | 20 ± 5.2 |
| 5 | Scop. + **9c*** | 0.5 + 0.5 | 37 | 17 | 20 ± 7.5 |
| 6 | Scop. + **9d** | 0.5 + 0.5 | 65 | 53 | 12 ± 5.8 |
| 7 | Scop. + **9e** | 0.5 + 0.5 | 66 | 53 | 13 ± 4.5 |
| 8 | Scop. + **9f** | 0.5 + 0.5 | 66 | 52 | 14 ± 5.6 |
| 9 | Scop. + **9g** | 0.5 + 0.5 | 64 | 47 | 17 ± 6.2 |
| 10 | Scop. + **9h** | 0.5 + 0.5 | 64 | 52 | 12 ± 5.5 |
| 11 | Scop. + Arecoline | 0.5 + 0.5 | 52 | 36 | 16 ± 5.5 |

Results are expressed as mean (±SEM), $n = 10$, $^{*}P < 0.05$ (Scop. = Scopolamine).

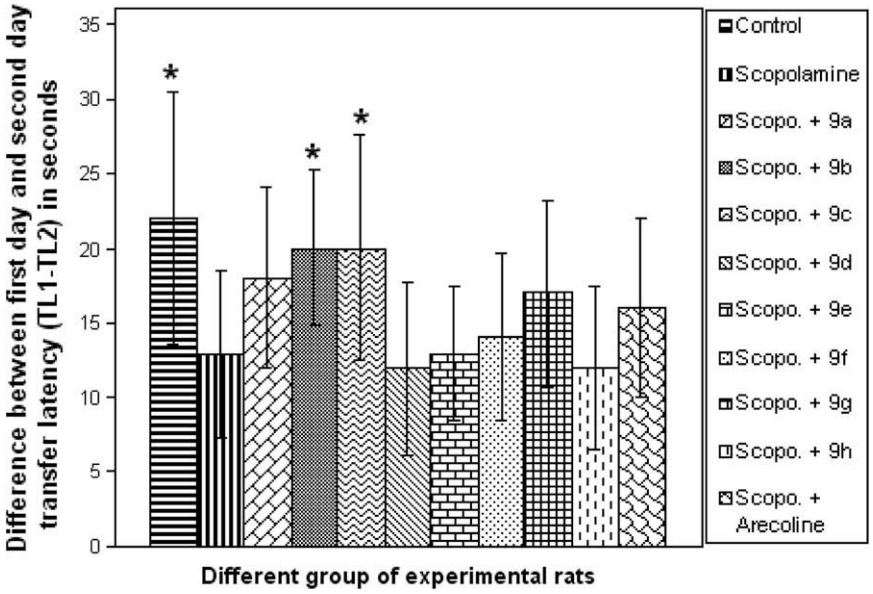



**Figure 6.** Effect of *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a**–**h**) on memory and learning against scopolamine induced memory loss and learning impairment on elevated plus maze in male Wistar rats ($^{*}p < 0.05$), mean (±SEM).

antidementia activity in vivo model tested. However, only **9b** could modulate the secretion of APP by activating M1 receptor in rat cerebrocortical slices, which might shift the proteolytic processing of APP towards the formation of non-amyloidogenic products and might reduces the production of Aβ. Consistent with our findings WAL 2014, a predominantly M1 agonist, induced APP secretion in cerebrocortical slices.[33] However, when the aryl group was disubstituted at 2 and 5 positions with chloro group (**9e**) or when *tert*-butyl group (**9d**) is present at the *para* position, it showed nil affinity for the M1 receptor. This may be due to steric hindrance.

Substitution of aryl group with nitro group once again decreased the affinity of molecules for the M1 receptor. Among the molecules substituted with nitro group, **9g** with nitro group at *meta* position showed good affinity, whereas nitro at *ortho* (**9f**) and at *para* (**9h**) positions showed least affinity for M1 receptor in vitro and average potency in reversing scopolamine induced memory loss in vivo. This might be due to electron withdrawing nature of $NO_2$ at *ortho* and at *para* positions of the aryl group or substitution at these two positions might hinder the binding of molecules at binding site of M1 receptor. However, $NO_2$ at *meta* position showed good affinity and potency compared to $NO_2$ at *ortho* and *para* positions, this may be due to less electron withdrawing nature of $NO_2$ at the *meta* position or fits better at the binding site. These *N*-aryl carboxamide substituted 3-morpholino arecoline **9**(**a–h**) derivatives showed no visible cholinergic toxicity at the dose tested (salivation, defecation, etc). One of the promising new derivatives (**9b**) would emerge as the potent molecule having antidementia activity.

## 4. Experimental

### 4.1. Chemistry

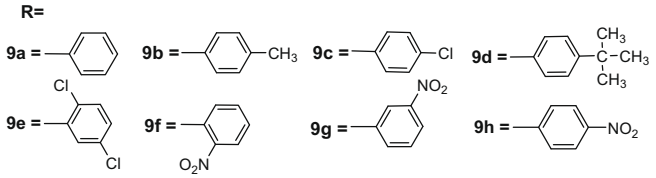
#### 4.1.1. General

The synthetic route utilized for the preparation of the compounds is shown in Scheme 1. The morpholino arecoline compounds **9**(**a–h**) were synthesized in total nine steps. Bromination of 3-acetylpyridine **1** with $Br_2$/HBr in glacial acetic acid gave the HBr salt of bromoacetylpyridine **2**. This was converted to amino alcohol **3**, by reaction with *N*-benzylaminoethanol in DMF in the presence of $K_2CO_3$. The keto group of compound **3** was reduced with $NaBH_4$ in methanol to offer the dihydroxy compound **4**. Treatment of compound **4** with 70% $H_2SO_4$ under reflux conditions caused dehydration to yield the cyclized product **5**. The *N*-benzyl group was removed by refluxing amine **5** in methanol in the presence of 10% Pd–C and ammonium formate. The resulting free amine was treated with Boc-anhydride in THF in the presence of $K_2CO_3$ to yield the Boc-protected compound **6**. This was converted to the corresponding methylamine hydroiodide salt by reaction with methyl iodide in acetone. This on treatment with sodium borohydride in methanol gave the reduced product **7**. Finally, the Boc group was removed using methanolic HCl to yield the free amine as HCl salt **8**. The detail procedure for the synthesis of intermediate compound **8** has been previously reported from our laboratory.[24] Compound **8** on reacting with the respective acid chlorides gave carboxamide compounds **9**(**a–h**). $^1$H NMR spectra of all compounds **9**(**a–h**) showed a multiplet at 8–7 due to aromatic protons and 5.7–5.8 due to alkene of tetrahydro pyridine. All the synthesized compounds were characterized by IR, $^1$H NMR, mass spectroscopy and CHNS analysis.

All chemicals and reagents were obtained from Aldrich (USA), Spectrochem Pvt. Ltd (India) and Rankem Pvt. Ltd (India) and were used without further purification. The I.R. spectra were recorded using Nujol on JASCO-FTTR, 41007 series. The $^1$H spectra were recorded on 400 MHz Bruker FT-NMR Spectrometer. The chemical shifts were reported as parts per million ($\delta$ ppm) tetramethyl silane (TMS) as an internal standard. Mass spectra were obtained on LCMSD-Trap-XCT instrument. Elemental analysis were performed on a variol EL, III Elementar C, H, N analyzer and values were within the acceptable limits of the calculated values. The progress of the reaction was monitored on pre coated silica gel plates (Merck) using chloroform/methanol (9:1) as a solvent system. Spectral data (IR, NMR and mass spectra) confirmed the structures of the synthesized compounds. Elemental (C, H and N) analysis indicated that the calculated and observed values were within the acceptable limits (±0.4%).

#### 4.1.2. General procedure for the synthesis of compounds 9(a–h)

The intermediate compound 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)morpholine (compound **8**) was synthesized as summarized in the Scheme 1 as per reported procedure (Kumar et al., 2007). To a stirred solution of compound **8** in dichloromethane, triethylamine (5.0 equiv) was added and cooled to 0–5 °C, then acid chloride (1.0 equiv) was added drop wise. The reaction mixture was stirred for 4–5 h at room temperature (completion of reaction was confirmed by TLC) then reaction mixture was washed with water, followed by saturated NaCl solution and dried over sodium sulfate. Dichloromethane was evaporated under reduced pressure and the crude residue obtained was purified by column chromatography using chloroform–methanol (9:1) as an eluent.




#### 4.1.3. (2-(1,2,5,6-Tetrahydro-1-methylpyridin-3-yl)morpholino)(phenyl)methanone (9a)

Compound **9a** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with benzoyl chloride (0.164 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 91%; IR (Nujol, $cm^{-1}$): 1670 (C=O), 1675 (–RC=CH–); $^1$H NMR ($CDCl_3$): $\delta$ 7.62–7.51 (m, 5H), 5.76 (br s, 1H, –C=C–), 3.85–3.79 (m, 3H), 3.49–3.47 (m, 1H), 2.78–2.75 (m, 3H), 2.77–2.75 (m, 2H), 2.54–2.48 (m, 2H), 2.23 (s, 3H), 2.09 (m, 2H). MS *m/z*: 387.4 ($M^+$). Anal. Calcd for $C_{17}H_{22}N_2O_2$: C, 71.30; H, 7.74; N, 9.78. Found: C, 71.13; H, 7.98; N, 9.51.

#### 4.1.4. (2-(1,2,5,6-Tetrahydro-1-methylpyridin-3-yl)-morpholino)(*p*-tolyl)methanone (9b)

Compound **9b** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 4-methylbenzoyl chloride (0.18 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 85%; IR (Nujol, $cm^{-1}$): 1670 (C=O), 1675 (–RC=CH–); $^1$H NMR ($CDCl_3$): $\delta$ 7.665–7.640 (d, 2H, $J$ = 8.9 Hz), 7.360–7.338 (d, 2H, $J$ = 8.80 Hz), 5.76 (br s, 1H, –C=C–), 3.85–3.72 (m, 3H), 3.50–3.45 (m, 1H), 2.91–2.89 (m, 3H), 2.79–2.76 (m, 2H), 2.52–2.43 (m, 2H), 2.22 (s, 3H), 2.13 (s, 3H), 2.02 (m, 2H). MS *m/z*: 301.4 ($M^+$); Anal. Calcd for $C_{18}H_{24}N_2O_2$ : C, 71.97; H, 8.05; N, 9.33. Found: C, 72.01; H, 8.03; N, 9.45.

#### 4.1.5. (4-Chlorophenyl) (2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)-morpholino)methanone (9c)

Compound **9c** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 4-chloro-benzoyl chloride (0.2 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 90%; IR (Nujol, $cm^{-1}$): 1675 (C=O), 1680 (–RC=CH–); $^1$H NMR ($CDCl_3$): $\delta$ 7.91–7.88 (m, 2H), 7.50 (m, 2H), 5.76 (br s,

1H, –C═C–), 3.83–3.79 (m,3H), 3.49–3.46 (m, 1H), 2.78–2.75 (m, 3H), 2.76–2.73 (m, 2H), 2.54–2.48 (m, 2H), 2.23 (s, 3H), 2.09 (m, 2H). MS *m/z*: 321.8 ($M^+$); Anal. Calcd for $C_{17}H_{21}ClN_2O_2$ : C, 63.64; H, 6.60; N, 8.73. Found: C, 64.15; H, 6.39; N, 8.55.

#### 4.1.6. (4-*tert*-Butylphenyl)(2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)-morpholino)methanone (9d)

Compound **9d** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 4-*tert*-butylbenzoyl chloride (0.230 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 87%; IR (Nujol, $cm^{-1}$): 1673 (C═O), 1678 (–RC═CH–); $^1H$ NMR ($CDCl_3$): $\delta$ 7.895–7.873 (d, 2H, $J$ = 8.77 Hz), 7.240–7.219 (d, 2H, $J$ = 8.73 Hz), 5.76 (br s, 1H, –C═C–), 3.83–3.76 (m, 3H), 3.49–3.44 (m, 1H), 2.89–2.85 (m, 3H), 2.77–2.75 (m, 2H), 2.54–2.48 (m, 2H), 2.24 (s, 3H), 2.08 (m, 2H), 1.02 (s, 9H). MS *m/z*: 343.5 ($M^+$); Anal. Calcd for $C_{21}H_{30}N_2O_2$: C, 73.65, H, 8.83, N, 8.18. Found: C, 73.83; H, 8.99; N, 8.35.

#### 4.1.7. (2,4-Dichlorophenyl)(2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)-morpholino)methanone (9e)

Compound **9e** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 2,5-dichlorobenzoyl chloride (0.244 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 88%; IR (Nujol, $cm^{-1}$): 1673 (C═O), 1680 (–RC═CH–); $^1H$ NMR ($CDCl_3$): $\delta$ 7.896–7.889 (d, 1H, $J$ = 2.0 Hz), 7.720–7.699 (d, 2H, $J$ = 8.12 Hz), 5.78 (br s, 1H, –C═C–), 3.83–3.72 (m, 3H), 3.49–3.44 (m, 1H), 2.78–2.76 (m, 3H), 2.79–2.76 (m, 2H), 2.54–2.43 (m, 2H), 2.22 (s, 3H), 2.02 (m, 2H). MS *m/z*: 356.3 ($M^+$); Anal. Calcd for $C_{17}H_{20}Cl_2N_2O_2$ : C, 57.47; H, 5.67; N, 7.89. Found: C, 57.62; H, 5.72; N, 7.67.

#### 4.1.8. (2-(1,2,5,6-Tetrahydro-1-methylpyridin-3-yl)morpholino)(2-nitrophenyl)methanone (9f)

Compound **9f** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 2-nitro benzoyl chloride (0.217 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 90%; IR (Nujol, $cm^{-1}$): 1670 (C═O), 1675 (–RC═CH–); $^1H$ NMR ($CDCl_3$): $\delta$ 8.364–8.343 (d, 1H, $J$ = 8.8 Hz), 8.125–8.102 (d, 1H, $J$ = 8.4 Hz), 7.87–7.85 (m, 2H), 5.78 (br s, 1H,–C═C–), 3.83–3.72 (m, 3H), 3.49–3.44 (m, 1H), 2.79–2.76 (m, 2H), 2.78–2.76 (m, 3H), 2.54–2.43 (m, 2H), 2.19 (s, 3H), 2.02 (m, 2H). MS *m/z*: 332.4 ($M^+$); Anal. Calcd for $C_{17}H_{21}N_3O_4$: C, 61.62; H, 6.39; N, 12.68. Found: C, 61.46; H, 6.45; N, 12.55.

#### 4.1.9. (2-(1,2,5,6-Tetrahydro-1-methylpyridin-3-yl)morpholino)(3-nitrophenyl)methanone (9g)

Compound **9g** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 3-nitro benzoyl chloride (0.217 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 85%; IR (Nujol, $cm^{-1}$): 1670 (C═O), 1675 (–RC═CH–); $^1H$ NMR ($CDCl_3$): $\delta$ 8.850–8.845 (d, 1H, $J$ = 1.2 Hz), 8.30–8.22 (m, 2H), 7.23 (m, 1H), 5.78 (br s, 1H, –C═C–), 3.85–3.81 (m, 3H), 3.49–3.44 (m, 1H), 2.79–2.76 (m, 2H), 2.78–2.76 (m, 3H), 2.55–2.45 (m, 2H), 2.22 (s, 3H), 2.02 (m, 2H). MS *m/z*: 332.3 ($M^+$); Anal. Calcd for $C_{17}H_{21}N_3O_4$: C, 61.62; H, 6.39; N, 12.68. Found: C, 62.05; H, 5.99; N, 12.25.

#### 4.1.10. (2-(1,2,5,6-Tetrahydro-1-methylpyridin-3-yl)morpholino)(4-nitrophenyl)methanone (9h)

Compound **9h** was obtained by reaction of compound **8** (0.2 g, 0.00078 mol) with 4-nitro benzoyl chloride (0.217 g, 0.0011 mol) and triethylamine (0.393 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 95%; IR (Nujol, $cm^{-1}$): 1670 (C═O), 1675 (–RC═CH–); $^1H$ NMR ($CDCl_3$): $\delta$ 8.324–8.299 (d, 2H, $J$ = 9.2 Hz), 7.931–7.908 (d, 2H, $J$ = 9.16 Hz), 5.76 (br s, 1H, –C═C–), 3.83–3.76 (m, 3H), 3.49–3.44 (m, 1H), 2.77–2.75 (m, 3H), 2.79–2.76 (m, 2H), 2.54–2.48 (m, 2H), 2.24 (s, 3H), 2.07 (m, 2H). MS *m/z*: 332.3 ($M^+$); Anal. Calcd for $C_{17}H_{21}N_3O_4$: C, 61.62; H, 6.39; N, 12.68. Found: C, 61.3; H, 6.78; N, 12.11.

### 4.2. Biology

#### 4.2.1. Displacement study

The competitive inhibition study was done using *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**) to find out their affinity towards cortical M1 receptor. Male Wistar rat brain cortex was used for synaptosomal membrane preparation. Crude membrane pellet was obtained from brain tissue, homogenized in 20 volumes of Tris–HCl buffer (50 mmol/l, pH 7.4) containing 0.32 mol/l sucrose, following the procedure described by Creese and Snyder.[34] The tissue homogenate was centrifuged at a speed of 1000*g* for 10 min at 4 °C, to remove cellular debris. The supernatant obtained was centrifuged at 32,000*g* for 20 min at 4 °C. Pellet obtained was resuspended in 50 mmol/l phosphate assay buffer (pH 7.4) containing 1 mmol $MgCl_2$. The protein concentration was estimated by method described by Lowry et al.[35]

The affinity of various compounds towards M1 receptor were estimated by using [$^3H$]QNB (0.2 nM, specific activity 48 Ci/mmol, Amersham, Little Chalfont, Bucks, UK) essentially following the procedure described by Hyttel et al.[36] and Yamamura and Snyder,[37] with slight modification. In brief an aliquot of synaptosomal membrane proteins (50 μg) was incubated with different concentration of compounds (0.1 μM–1 mM) as a displacer and [$^3H$]QNB (0.2 nM) and reaction volume was made up to 200 μl with assay buffer and incubated for 2 h at 37 °C. The reaction for all displacement assay was stopped by adding ice-cold assay buffer and reaction mixtures were rapidly filtered through GF/B filters under vacuum. The filters were transferred to vials containing scintillation fluid, (5 ml) and allowed to equilibrate overnight. Radioactivity was measured in a liquid scintillation counter (Tris-Carb 2100TR, Packard, US) at 65% efficiency. The data from displacement were analyzed and $IC_{50}$ and $K_i$ values are obtained from LIGAND–DRUG programme.[38] The mean values of % bound are plotted against log of displacer concentration.

#### 4.2.2. IP3 levels estimation

Inositol (1,4,5)-trisphosphate (IP3) levels were estimated using [$^3H$]myoinositol (s.a. 16.0 Ci/mmol, Amersham) in rat cerebral cortex slices, at basal level and also after stimulation by different concentrations of synthesized *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**) (1 μM–1 mM), to find out their efficacy and potency to elevate the IP3 formation, by comparing it with basal IP3 levels and IP3 formation elevated by acetylcholine iodide, following the procedure described by Kendall and Naharoski;[39] Van Rooijen et al.,[40] and Gonzales et al.,[41] with slight modification. In brief: Cross chopped (300 × 300 μm) rat brain cerebrocortical slices (0.5 g) were prepared and incubated in oxygenated Kreb's Ringer bicarbonate (KRB) buffer (pH 7.4) with 0.5 μCi [$^3H$]myoinositol for 1 h at 37 °C. Tissues were washed with the same buffer and incubated with LiCl (10 mM). Assay was terminated by the addition of 10% trichloracetic acid (TCA). Samples were kept on ice for 20 min and homogenized and sedimented by centrifugation at 3000*g*. Supernatant was washed 4–5 times with water saturated diethyl ether and neutralized with $NaHCO_3$ (5 mM). Dowex-1-chloride (AG-X8: 200-400 mesh size) resin was used for separation of [$^3H$]IP3 form the reaction mixture by anion-exchange chromatography. The bound [$^3H$] IP3 was eluted from the column using 0.8 M formate and 0.1 M formic acid. The radioactivity of [$^3H$]IP3 formed was measured in a liquid scintillation counter at 65% efficiency. The levels of IP3 were expressed as picomoles of [$^3H$]IP3 formed per milligram of protein.

#### 4.2.3. Modulation of APP Secretion in rat brain cerebrocortical slices

Rat cerebral cortex was dissected out and minced in two perpendicular directions by McIlwain tissue chopper (300 × 300 μm) and rinsed three times with oxygenated (95% $O_2$/5% $CO_2$) Krebs Ringer Bicarbonate buffer (pH 7.4) consisting of (120 mM NaCl; 4.65 mM KCl; 1.2 mM $MgSO_4$; 1.2 mM KH $PO_4$; 11.2 mM glucose; 25 mM $NaHCO_3$; 2.5 mM $CaCl_2$). Washed slices were equilibrated for 50 min at 37 °C under oxygen atmosphere. The buffer was then changed to a fresh Krebs buffer containing 50 μg/ml bovine serum albumin and a cocktail of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin and 5 units/ml aprotinin. Aliquots of the slices were transferred to Eppendorff tubes (50 mg slices/0.5 ml) and stimulated for 1 h at 37 °C with the tested compounds while oxygenating and gently stirring the slices every few minute. At the end of the incubation period, the medium was collected for secreted APP determinations.

Protein content of the collected medium was measured by method described by Lowery et al. (1953), and loaded (50 μg/lane) on 10% SDS–PAGE (BioRad). The gel was run at 35 mA, 100 W for 2 h. Proteins were then transferred on to a nitrocellulose membrane (Millipore) using a semidry transfer unit (BioRad). Membrane was blocked in 5% non-fat milk powder in PBS-T (0.1% Tween 20 in PBS). The membrane was incubated with primary monoclonal antibody 22C11 (anti-Alzheimer precursor protein A4; Chemicon/Millipore) of the appropriate concentration for 2 h. The membrane was then washed 3 times for 10 min each on a rocker with Phosphate Buffer Saline (PBS), incubated with an appropriate horse radish peroxidase conjugated secondary antibody for 1 h and then sequentially washed 2 × 2 min, 1 × 15 min and finally 3 × 5 min with PBS-T. For APP detection, the Renaissance Chemiluminescence Reagent (PerkinElmer Life Sciences) was employed followed by exposure to an autoradiography film (Hyperfilm-ECL; Amersham). Signal intensities were quantified using SCION IMAGE software.

#### 4.2.4. Antiamnesic activity

It was carried out for synthesized *N*-aryl carboxamide substituted 3-morpholino arecoline derivatives **9**(**a–h**) against scopolamine induced memory loss using passive avoidance step down task paradigm in male Wistar rats weighing 200–250 g ($n = 10$) according to the method described by Sharma and Kulkarni.[42,43]

#### 4.2.5. Elevated plus maze

The elevated plus maze experiments were done to elucidate the effect of our synthesized molecules on memory and learning against scopolamine induced memory loss in rats by following the procedure described by Itoh et al.[44] and also by Sharma and Kulkarni[45] with slight modification. In brief: This was employed for the measurement of transfer latency (TL). The male Wistar rats (weighing 200–250 g, $n = 10$) were selected, grouped and were administered scopolamine and test compound along with scopolamine to respective animal groups. Thirty minutes later they were placed individually at the end of one arm facing away from the central platform and the time they take to move from open arm to either of enclosed arms (TL) was measured. On the 1st day male Wistar rats were allowed to explore the plus maze for 90 s. Control group animals were treated with saline (0.9%) and on 2nd day TL was measured in the similar way on the same animals. The resultant data were subjected to statistical analysis.[43]

#### 4.2.6. Acute toxicity

Rats (6 per group), which had fasted 16 h, were treated orally with various doses of the compounds and observed for 1 week after treatment, deaths were recorded daily. None of the rats died within one week after administration under test dose.

#### 4.2.7. Dose–response curve

Different doses (0.05–2 mg/kg of the body wt.) of the derivatives were selected to find out optimum dose (found to be 0.5 mg/kg) for in vivo studies.

### 5. Data analysis

The data from the displacement assay was analyzed using 'LIGAND–DRUG' software programme[38] to obtain the $IC_{50}$ and $K_i$ values (both are expressed in μmol). All the data are expressed as mean ± SD. The statistical analysis was done by using student's *t*-test. Differences were considered to be significant at $P < 0.05$. All analysis were performed with the 'JANDEL-SCIENTIFIC-SIGMA STAT' software, version 2.0 for windows.

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