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

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ABSTRACT

As part of our continuing effort aimed at the development of selective, efficacious and centrally active M1 muscarinic agonists for the treatment of Alzheimer's presenile dementia, a series of *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9(a–j)** were synthesized by using *N*-benzyl amino ethanol coupling with α-bromo acetyl pyridine followed by reduction and cyclization to develop a new class of M1 receptor agonists. Subsequently the synthesized compounds were subjected to in vitro radioligand M1 receptor affinity studies, IP3 formation studies and also to in vivo pharmacological evaluation of memory and learning in male Wistar rats. Derivatives with chloro (**9f**) and methoxy (**9c**) groups on the para position of the benzene ring attached to the nitrogen of thiourea showed several fold high affinity for the M1 receptor (in vitro) among all the synthesized molecules **9**(**a–j**), and also significantly elevated IP3 levels and as well elicited beneficial effects in vivo in memory and learning models in rats (rodent memory evaluation, plus and Y maze studies).

1. Introduction

Neurochemical examination of the brain material from Alzheimer's patients has demonstrated the loss of presynaptic marker enzyme choline acetyltransferase and the muscarinic receptors of the M2 subtype, which are mainly responsible in causing deficits in central cholinergic transmission in Alzheimer's patients.[1–3] The postsynaptic muscarinic receptors, which primarily are of the M1 subtype, to a large extent, seem to survive the loss of cholinergic nerve endings.[4] These findings have led to attempts at restoring cholinergic function by means of cholinomimetic drugs such as muscarinic agonists, acetylcholinesterase inhibitors and acetylcholine releasing agents, the hypothesis being that enhancement of cholinergic neurotransmission would alleviate the symptoms of the diseases, particularly the deficits in cognition and memory.[4] Four types of muscarinic receptors are known, named M1 to M4,[5] and five subtypes of muscarinic receptors have been cloned and designated m1–m5.[6,7] Identifying M1 selective muscarinic agonists, which are capable of crossing the blood–brain barrier is the subject of active research for pharmacological application.[8]

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. [9] The lack of M1 selectivity and efficacy due to dose limiting side effects associated with M2 and M3 muscarinic receptor subtype stimulation has produced disappointing results.[9] Replacement of the ester functionality of arecoline with either the 3-alkoxy-1,2,5 thiadiazole[8] or the 3-alkyl-1,2,4-oxadiazole[10] 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 also had lower affinities for muscarinic receptors;[10] see the structure of Arecoline and its derivatives (Fig. 1). No muscarinic M1 subtype selectivity has been reported for 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)morpholine. C-Functionalized morpholines are found in various naturally occurring products as well as in drugs.[11] Since compound 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl) morpholine is a conformationally restricted arecoline analogue, we were encouraged to pursue this compound.

In our previous studies, we have reported arecoline thiazolidinones[12] and *N*-arylsulphonamide substituted 3-morpholino arecoline derivatives[13] as muscarinic receptor 1 agonist. In a continued effort to discover less toxic arecoline class of muscarinic agonist

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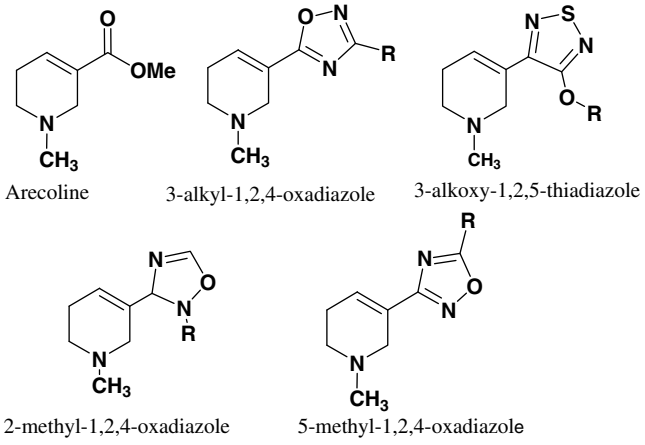
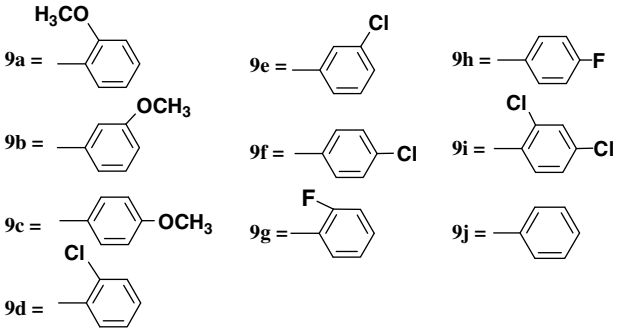


Figure 1. Arecoline and arecoline derivatives.

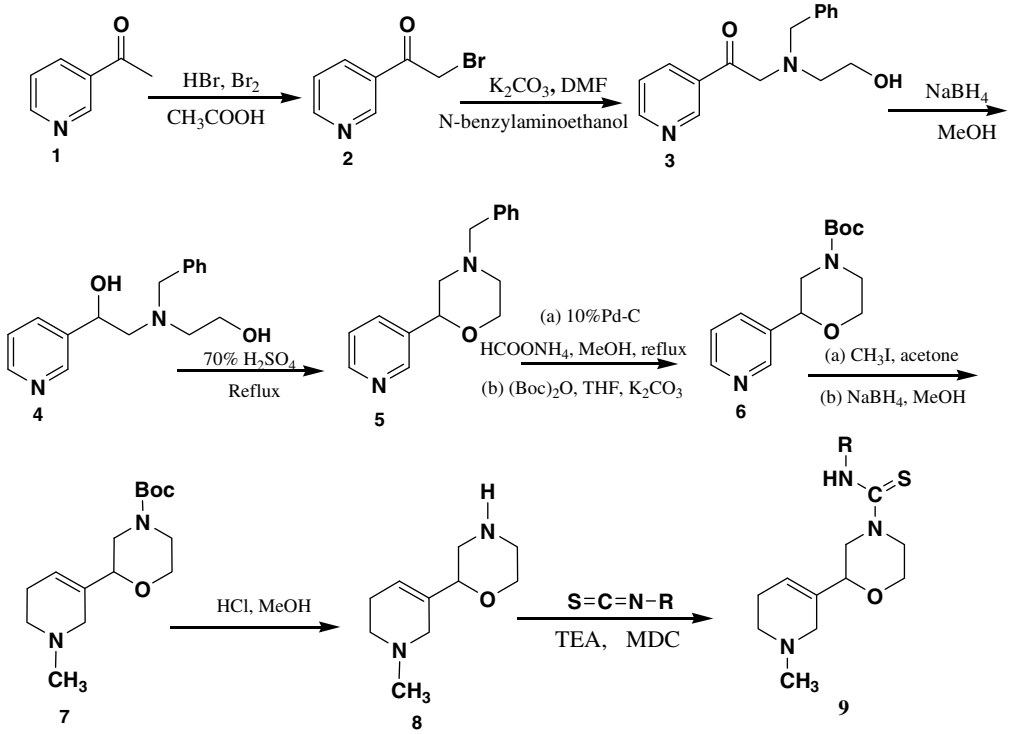
and to further improve their selectivity and potency, here, we are reporting our findings of *N*-arylthioureas substituted 3-morpholino arecoline derivatives [**9(a–j)**, scheme 1] along with their in vitro muscarinic binding studies by using [^{3}H]QNB with male Wistar rat brain synaptosomal membrane, IP3 levels estimation stimulated by the synthesized molecules **9(a–j)** and also in vivo evaluation of memory and learning in male Wistar rats, 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 affinity assay for the synthesized *N*-arylthioureas substituted 3-morpholino arecoline **9(a–j)** derivatives (Scheme 1). Four derivatives among all synthesized compounds **9**(**a–j**) showed greater affinity and potency towards the M1 receptor (Table 1 and Fig. 2), which is in the order of **9f** > **9c** > **9h** > **9g**. The most potent compound among all tested derivatives, **9f** (K_i = 0.31 μM), is one with chlorine, at the para position of the aryl group attached to the nitrogen of thiourea. Substitution of methoxy group (**9c**), at para position of the aryl group also showed good affinity towards the M1 receptor in vitro. However, when chloro and methoxy groups are substituted at meta position (**9e** and **9b,** respectively) they showed moderate affinity, and when they are substituted at ortho position (**9d** and **9a,** respectively) they showed least or no affinity, respectively, for the M1 receptor in vitro. In similar fashion, introduction of fluoro group at para position (**9h**) also showed good affinity for the M1 receptor, whereas when introduced at ortho position (**9g**) it decreases the affinity of compound for the receptor in vitro. However, disubstituted chlorine (**9i**) at ortho and para positions showed only moderate affinity for the receptor. Compound **9j** with benzene (without any substitution on it) ring substituted on the nitrogen of thiourea showed average affinity towards the M1 receptor in vitro.



Agonist stimulation of the M1 receptor results in a rapid elevation of IP3 levels.[14] Therefore, we planned to measure the efficacy and



Scheme 1.

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

Compounds	K_i (μM)	IC_{50} (μM)
9a	98 ± 12.36	362 ± 17.47
9b	41 ± 9.08	120 ± 4.21
9c	**4 ± 0.36**	**15 ± 2.73**
9d	214 ± 21.92	515 ± 28.69
9e	85 ± 6.98	236 ± 11.87
9f	**0.31 ± 0.12**	**1.2 ± 0.25**
9g	15 ± 3.36	67 ± 10.56
9h	**7 ± 1.28**	**31 ± 5.12**
9i	31 ± 4.90	112 ± 08.16
9j	49 ± 5.36	153 ± 8.59
Arecoline	86 ± 8.39	469 ± 17.54

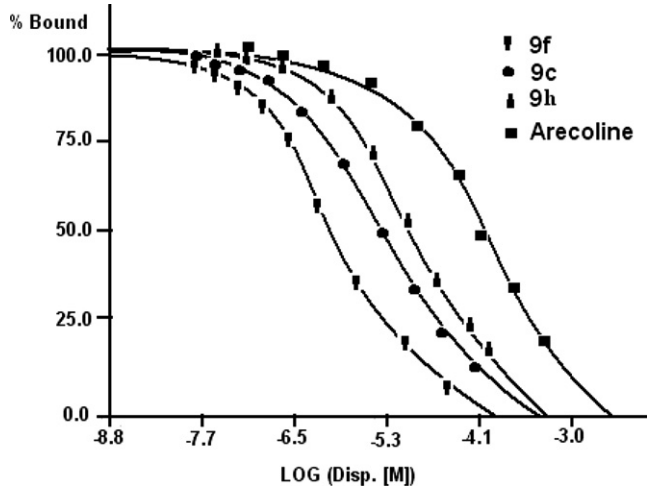


Figure 2. Displacement graphs of three potent compounds **9f**, **9c** and **9h**. The displacement studies were done with 0.2 nM [^{3}H]QNB and different concentrations of *N*-arylthioureas substituted 3-morpholino arecolines **9**(**a**–**j**). The mean values of% bound are plotted against log of displacer concentration. IC_{50} and Ki values are obtained from Ligand–Drug programme.

potency of synthesized compounds **9(a–j)** to elevate the IP3 formation in rat cerebral cortex slices. Basal IP3 levels was found to be 0.61 pmol/mg protein, and it was significantly increased by all the four derivatives (**9f, 9c**, **9h** and **9g**), which were showing high affinity for M1 receptor in binding studies. The potency of these compounds to elevate the basal IP3 levels was compared with that of IP3 levels elevated by acetylcholine iodide, which represents M1 receptor-linked PLC activity (Table 2 and Fig. 3).

It has been demonstrated that the M1 receptor subtypes predominate in the cerebral cortex and hippocampus areas[15,16] in which cholinergic transmission appears to be essential for learning and memory. The foresaid in vitro M1 receptor binding studies and subsequently IP3 formation studies formed a basis for extending correlation further to in vivo pharmacological studies to ascertain applicability of the synthesized *N*-arylthioureas substituted 3-morpholino arecoline **9(a–j)** derivatives in scopolamine (muscarinic antagonist)-induced dementia models using memory and learning experiments (passive avoidance tasks, plus and Y maze studies). In accordance with the degree of affinity and potency of synthesized molecules **9(a–j)** in vitro binding experiments elicited almost anticipated level of pharmacological actions in reversing scopolamine-induced dementia (Fig. 4). Three synthesized morpholino arecoline derivatives **9f**, **9c** and **9h** were best among all **9(a–h)** synthesized compounds in reversing scopolamine-induced dementia by making rats to commit less number of mistakes (Number of mistakes done 10, 10 and 11, respectively) when compared with

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

Rat-group	IP3 Conc. (pM)	Stimulation (%)
Control (Basal)	0.61 ± 0.05	—
9a-Stimulated	0.61 ± 0.02	100
9b*-Stimulated	0.65 ± 0.06	106
9c*-Stimulated	1.09 ± 0.05	178
9d-Stimulated	0.61 ± 0.06	100
9e-Stimulated	0.63 ± 0.05	103
9f*-Stimulated	1.32 ± 0.06	216
9g*-Stimulated	0.79 ± 0.03	129
9h*-Stimulated	0.95 ± 0.03	155
9i-Stimulated	0.71 ± 0.04	116
9j-Stimulated	0.62 ± 0.04	101
Acetylcholine*-Stimulated	1.64 ± 0.06	268

The efficacy of novel *N*-arylthioureas substituted 3-morpholino arecoline **9**(**a**–**j**) derivatives (100 μmol/l) for the M1 receptor was measured by its 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 [3H]IP3 formed per milligram of tissue. $n = 6$, $^*P = <0.001$.

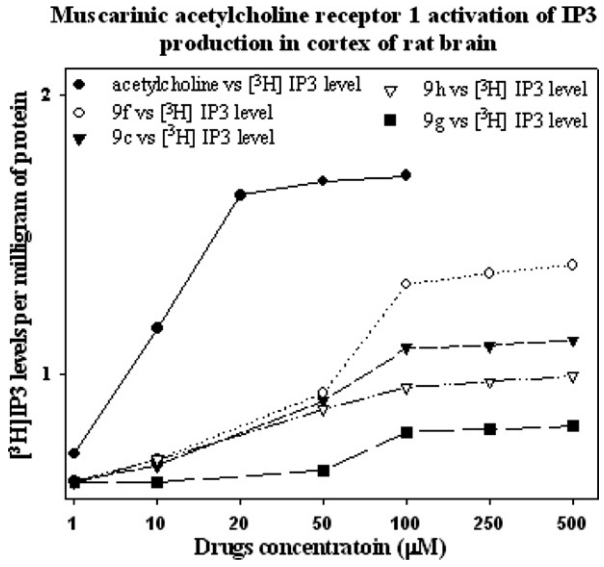


Figure 3. The scatchard graph of four potent compounds (**9f, 9c, 9h** and **9g**) among all the tested derivatives **9(a–j)**. Inositol (1,4,5)-trisphosphate (IP3) levels were estimated using [^{3}H]myoinositol, in rat cerebral cortex slices after the stimulation by different concentrations of *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9(a–j),** and significance was compared with the basal IP3 levels, and their potency to elevate the IP3 formation was determined by comparing it with IP3 levels elevated by acetylcholine iodide as described in Section 4. Data points are the means of three experiments, each assayed in duplicate.

the number of mistakes done by control rats (8 mistakes) and scopolamine-treated group (33 mistakes). Compounds **9g**, **9b** and **9i** also significantly reversed the scopolamine-induced memory loss. Compounds **9d, 9a** and **9e** were the least potent compounds among all tested derivatives.

The in vivo plus maze for synthesized derivatives **9**(**a**–**j**) in male Wistar rats measures the transfer latency (TL) in seconds taken to reach from one extreme ends of open arm also to one of the closed arms in a plus maze. Difference in TL in seconds between 1st day and 2nd day for scopolamine-treated group and test compound along with scopolamine-treated groups was compared to evaluate learning (TL1) and memory (TL2). Derivatives **9f**, **9c** and **9h** significantly reversed acute memory loss and learning impairment in

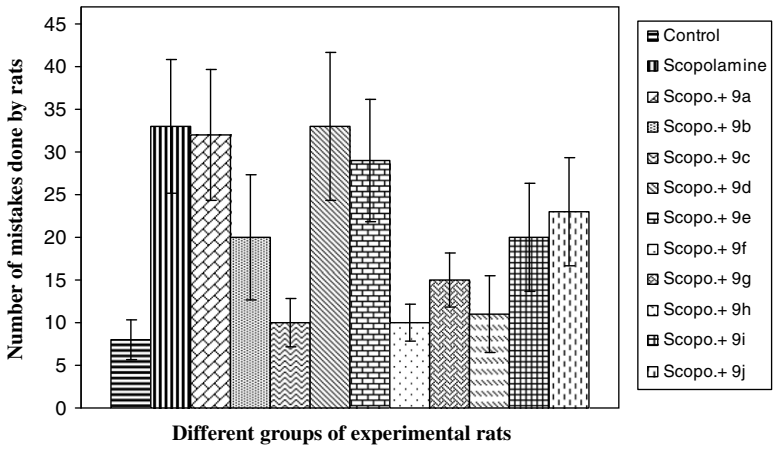


Figure 4. Antiamnesic effect of *N*-arylthioureas substituted 3-morpholino arecolines **9(a–j)** against scopolamine-induced memory loss in male Wistar rats. Mean (± SEM).

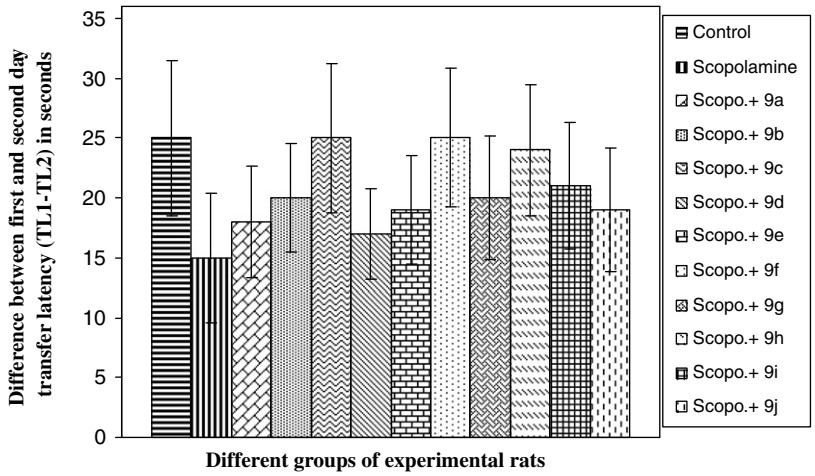


Figure 5. Effect of *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9**(**a–j**) on memory and learning against scopolamine-induced memory loss and learning impairment on elevated plus maze in male Wistar rats. Mean (± SEM).

male Wistar rats (Fig. 5). **9f** produced lesser TL for 1st day (TL1 = 34 s) compared to scopolamine-treated group (TL1 = 68 s), but on 2nd day (TL2 = 9 s) TL was lesser than 1st day (**9f**) indicating how it is helpful in reversing learning impairment as compared to rest of the derivatives. Overall difference between TL2 and TL1 was same for **9f**, **9c** and control groups (25 s). In contrary to this, compound **9d** produced longer 1st day TL (TL1 = 60 s) and 2nd day TL (TL2 = 43 s) overall difference differs (TL1–TL2 = 17 s), implying that the derivative fails to reverse acute memory and learning impairing in male Wistar rats. TL for the remaining compounds is given in Figure 5.

The synthesized derivatives were also subjected to in vivo Y maze studies using male Wistar rats. Male Wistar rats were allowed to explore three-arm Y maze in which rats move in sequence. The percentage of alteration behaviour an animal does by not entering into the already entered arm before completing the sequence was noted. The alteration behaviour in terms of percentage of alteration behaviour was noted for test compounds in presence of scopolamine and was compared with scopolamine administered animal group (Fig. 6). The percentage of alteration behaviour for **9f**, **9h**, **9c** and **9g** (64, 61, 60 and 58%) indicates that derivatives significantly reverse scopolamine-induced spatial working memory loss (in terms of alteration behaviour). For the rest of the derivatives percentage of alteration behaviour is shown in Figure 6.

3. Conclusion

In light of these findings, in vitro competitive M1 receptor affinity assay; stimulation of IP3 formation studies and in vivo pharmacological experiments for the synthesized compounds **9(a–j),** to test the relation between affinity of these molecules to M1 receptor and their ability to reverse scopolamine-induced memory loss and learning impairment, have ascertained their applicability in dementia. We can conclude that position of substitutions on the benzene ring attached to the nitrogen of thioureas determines the affinity of the compound for the M1 receptor. The compound **9j,** which is not having any substitute group on the benzene attached to the nitrogen of thiourea, is found to have moderate affinity for the M1 receptor. But when chloro (**9f**) and methoxy (**9c**) are present at the para position of the benzene, it increases the affinity of the compounds several folds for the M1 receptor in vitro, and also showed greater potency in elevating the IP3 levels in rat cerebral slices and useful antidementia activity in the in vivo model tested. However, when the same groups are present at meta (**9e** and **9b,** respectively) or at ortho position (**9d** and **9a,** respectively), it decreases the affinity of compounds for the M1 receptor. In the similar fashion, when fluoro group is present at para position (**9h**) it increases the affinity of compound for the receptor but, when present at ortho position (**9g**) it decreases the affinity of compound for the M1 receptor. Decrease in the affinity of the com-

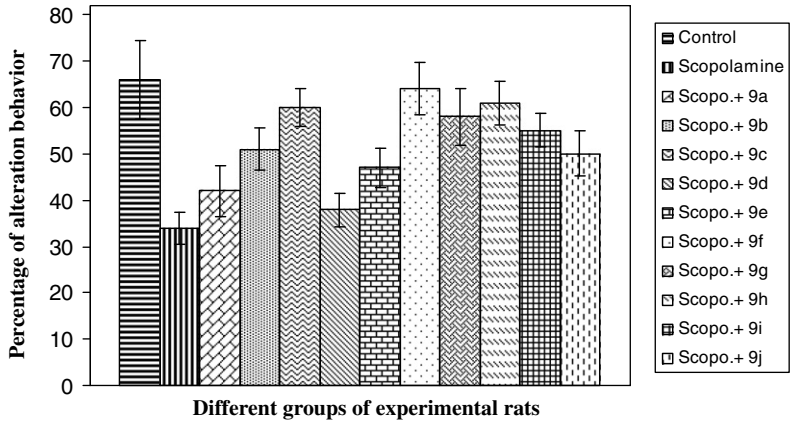


Figure 6. Effect of *N*-aryl thioureas substituted 3-morpholino arecoline derivatives **9**(**a**–**j**) on spatial working memory using Y maze task in male Wistar rats. Mean (± SEM).

pounds having substitute at ortho position may be due to the hindrance in binding to the active site of M1 receptor. Compound **9i** having disubstituted chlorine at ortho and para positions showed moderate affinity for the M1 receptor in vitro and average potency in reversing scopolamine-induced memory loss in vivo. These *N*-aryl morpholino arecoline derivatives **9(a–j)** showed no visible cholinergic toxicity at the dose tested (salivation, defecation, etc.). One of the promising new derivatives would emerge as the potent molecule having antidementia activity.

4. Experimental procedure

4.1. Chemistry

The *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9(a–j)** were synthesized in nine steps as shown in the scheme 1. 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 using $NaBH_4$ in methanol to obtain 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, and 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 detailed procedure for the synthesis of compound **8** has been reported in our previous paper.[17] This on reacting with respective isothiocyanates gave *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9**(**a–j**). ^{1}H NMR spectra of all compounds **9(a–j)** showed a multiplet at 9–7 due to aromatic protons and 5.7–5.8 due to double bond of tetrahydro pyridine. All the synthesized compounds were characterized by IR, ^{1}H NMR, Mass spectroscopy and CHNS analysis.

Infrared (IR) spectra were recorded using Nujol on JASCO-FTIR, 4100 series. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Shimadzu AMX 400-Bruker, 400MHz spectrometer using DMSO as a solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Mass and purity were recorded on a LC-MSD-Trap-XCT. Elemental (CHNS) analyses were obtained on Vario EL III Elementar. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates. All chemicals and reagents were obtained from Aldrich (USA), Spectrochem Pvt. Ltd. (India) and Rankem Pvt. Ltd. (India), and were used without further purification.

4.1.1. General procedure for the synthesis of *N*-arylthioureas substituted 3-morpholino arecoline derivatives 9(a–j)

The intermediate compound 2-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)morpholine (compound **8**, scheme 1) was synthesized by eight step reactions according to the previously described procedure.[17] To the stirred solution of compound **8** (1 equiv) in dichloromethane, triethylamine (5 equiv) was added and cooled to 0 °C, respective isothiocyanates (1 equiv) were added at cold condition and stirred at room temperature for 4–5 h (completion of reaction was confirmed by TLC). Reaction mixture was quenched using water and extracted using dichloromethane. The combined dichloromethane layer was washed with brine solution and dried over anhydrous sodium sulfate. Dichloromethane was evaporated under reduced pressure, and the crude product obtained was purified by silica gel (60–120 mesh) column. The compounds **9(a–j)** were eluted at 8% to 10% methanol in chloroform.

4.1.2. Synthesis of 2-(1,2,5,6-tetrahydro-1-methylpyridin-3yl)-*N*–(2-methoxyphenyl)morpholine-4-carbothioamide (9a)

The compound (**9a**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 2-methoxy phenyl isothiocyanate (0.162 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 82%; IR (Nujol, cm^{-1}): 3201(–NH–), 1389 (–CS–N–), 1675 (–RC═CH–). ^{1}H NMR ($CDCl_3$): δ 9.23(s, 1H), 7.16–7.14 (m, 1H), 6.88–6.86 (m, 2H), 6.784–6.763 (d, 1H, J = 8.4 Hz), 5.76 (br s, 1H, –C═CH–), 3.85–3.74 (m, 3H), 3.73 (s, 3H), 3.52–3.48 (m, 1H), 3.31–3.26 (m, 3H), 2.80–2.76 (m, 2H), 2.52–2.48 (m, 2H), 2.21 (s, 3H), 2.02 (m, 2H). MS (*m/z*): 348.53 (M^+).

4.1.3. Synthesis of 2-(1,2,5,6-tetrahydro-1-methylpyridin-3yl)-*N*–(3-methoxyphenyl)morpholine-4-carbothioamide (9b)

The compound (**9b**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 3-methoxy phenyl isothiocyanate (0.162 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 83%; IR (Nujol, cm^{-1}): 3206(–NH–), 1398 (–CS–N–), 1676 (–RC═CH–). ^{1}H NMR ($CDCl_3$): δ 9.23 (s, 1H), 7.19–7.16 (m, 1H), 6.89–6.84 (m, 2H), 6.677–6.657 (dd, 1H, J = 1.6 and 1.6 Hz), 5.74 (br s, 1H, –C═CH–), 3.84–3.73 (m, 3H), 3.70 (s, 3H), 3.53–3.49 (m, 1H), 3.33–3.28 (m, 3H), 2.81–2.76 (m, 2H), 2.52–2.47 (m, 2H), 2.23 (s, 3H), 2.01 (m, 2H). MS (*m/z*): 348.51 (M^+).

4.1.4. Synthesis of 2-(1,2,5,6-tetrahydro-1-methylpyridin-3yl)-*N*–(4-methoxyphenyl)morpholine-4-carbothioamide (9c)

The compound (**9c**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 4-methoxy phenyl isothiocyanate (0.162 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 86%; IR (Nujol, cm^{-1}): 3204 (–NH–), 1386 (–CS–N–), 1678 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.20 (s, 1H), 7.141–7.119 (d, 2H, J = 8.8 Hz), 6.863–6.842 (d, 2H, J = 8.4 Hz), 5.78 (bs, 1H, –C═CH–), 3.85–3.74 (m, 3H), 3.74 (s, 3H), 3.52–3.47 (m, 1H), 3.33–3.28 (m, 3H), 2.84–2.79 (m, 2H), 2.53–2.48 (m, 2H), 2.21 (s, 3H), 2.00 (m, 2H). MS (*m/z*): 348.51 (M^+).

4.1.5. Synthesis of *N*-(2-chlorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9d)

The compound (**9d**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 2-chloro phenyl isothiocyanate (0.166 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml).Yield: 88%; IR (Nujol, cm^{-1}): 3221 (–NH–), 1389 (–CS–N–), 1678 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.17 (s, 1H), 7.473–7.454 (d, 1H, J = 7.6 Hz), 7.25–7.19 (m, 3H), 5.73 (bs, 1H, –C═CH–), 3.80–3.75 (m, 3H), 3.53–3.50 (m, 1H), 3.30–3.23 (m, 3H), 2.80–2.72 (m, 2H), 2.54–2.46 (m, 2H), 2.26 (s, 3H), 2.05 (m, 2H). MS (*m/z*): 352.90 (M^+).

4.1.6. Synthesis of *N*-(3-chlorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9e)

The compound (**9e**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 3-chloro phenyl isothiocyanate (0.166 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 92%; IR (Nujol, cm^{-1}): 3224(–NH–), 1408 (–CS–N–), 1677 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.36 (s, 1H), 7.395–7.390 (d, 1H, J = 2.06 Hz), 7.27–7.20 (m, 2H), 7.133–7.116 (d, 1H, J = 7.8 Hz), 5.73 (bs, 1H, –C═CH–), 3.84–3.78 (m, 3H), 3.50–3.48 (m, 1H), 3.33–3.27 (m, 3H), 2.80–2.75 (m, 2H), 2.53–2.48 (m, 2H), 2.24 (s, 3H), 2.04 (m, 2H). MS (*m/z*): 352.90 (M^+).

4.1.7. Synthesis of *N*-(4-chlorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9f)

The compound (**9f**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 4-chloro phenyl isothiocyanate (0.166 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 92%; IR (Nujol, cm^{-1}): 3221 (–NH–), 1383 (–CS–N–), 1669 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.04 (s, 1H), 7.351–7.330 (d, 2H, J = 8.4 Hz), 7.234–7.215 (d, 2H, J = 7.6 Hz), 5.75 (bs, 1H, –C═CH–), 3.84–3.79 (m, 3H), 3.53–3.48 (m, 1H), 3.33–3.28 (m, 3H), 2.81–2.75 (m, 2H), 2.55–2.48 (m, 2H), 2.28 (s, 3H), 2.05 (m, 2H). MS (*m/z*): 352.90 (M^+).

4.1.8. Synthesis of *N*-(2-fluorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9g)

The compound (**9g**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 2-fluoro phenyl isothiocyanate (0.150 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 90%; IR (Nujol, cm^{-1}): 3232 (–NH–), 1392 (–CS–N–), 1675 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.13 (s, 1H), 7.464–7.442 (d, 1H, J = 8.8 Hz), 7.31–7.29 (m, 2H), 7.18–7.16 (m, 1H), 5.73 (bs, 1H, –C═CH–), 3.85–3.75 (m, 3H), 3.53–3.48 (m, 1H), 3.33–3.27 (m, 3H), 2.81–2.76 (m, 2H), 2.54–2.49 (m, 2H), 2.24 (s, 3H), 2.04 (m, 2H). MS (*m/z*): 336.48 (M^+).

4.1.9. Synthesis of *N*-(4-fluorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9h)

The compound (**9h**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 4-fluoro phenyl isothiocyanate (0.150 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 94%; IR (Nujol, cm^{-1}): 3243 (–NH–), 1394 (–CS–N–), 1676 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.02 (s, 1H), 7.419–7.404 (d, 2H, J = 7.20 Hz), 7.072–7.041 (d, 2H, J = 6.02 Hz), 5.74 (bs, 1H, –C═CH–), 3.85–3.75 (m, 3H), 3.50–3.48 (m, 1H), 3.31–3.23 (m, 3H), 2.80–2.74 (m, 2H), 2.54–2.46 (m, 2H), 2.24 (s, 3H), 2.05 (m, 2H). MS (*m/z*): 336.48 (M^+).

4.1.10. Synthesis of *N*-(2,4-dichlorophenyl)-2-(1,2,5,6-tetrahydro-1-methylpyridin-3-yl)morpholine-4-carbothioamide (9i)

The compound (**9i**) was obtained by the reaction of compound **8** (0.25 g, 0.00098 mol) with 2,4-dichloro phenyl isothiocyanate (0.20 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 87%; IR (Nujol, cm^{-1}): 3211 (–NH–), 1382 (–CS–N–), 1676 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.06 (s, 1H), 7.596–7.591 (d, 1H, J = 2.0 Hz), 7.368–7.347 (d, 1H, J = 8.4 Hz), 7.513–7.492 (d, 1H, J = 8.4 Hz), 5.70 (bs, 1H, –C═CH–), 3.83–3.78 (m, 3H), 3.54–3.50 (m, 1H), 3.32–3.24 (m, 3H), 2.80–2.74 (m, 2H), 2.54–2.46 (m, 2H), 2.25 (s, 3H), 2.08 (m, 2H). MS (*m/z*): 387.36 (M^+).

4.1.11. Synthesis of 2-(1,2,5,6-tetrahydro-1-methylpyridin-3yl)-*N*-phenylmorpholine-4-carbothioamide (9j)

The compound (**9j**) was obtained by the reaction of compound **8** (0.2 g, 0.00098 mol) with phenylisothiocyanate (0.132 g, 0.00098 mol) and triethylamine (0.394 g, 0.0039 mol) in dichloromethane (3 ml). Yield: 89%; IR (Nujol, cm^{-1}): 3212 (–NH–), 1389 (–CS–N–), 1674 (–RC═CH–). ^1H NMR ($CDCl_3$): δ 9.20 (s, 1H), 7.13–7.06 (m, 2H), 6.95–6.90 (m, 3H), 5.76 (b, 1H, –C═CH–), 3.85–3.76 (m, 3H), 3.53–3.49 (m, 1H), 3.32–3.28 (m, 3H), 2.79–2.78 (m, 2H), 2.53–2.48 (m, 2H), 2.20 (s, 3H), 2.04 (m, 2H), 1.24 (m, 3H). MS (*m/z*): 318.48 (M^+).

4.2. Biology

4.2.1. Displacement study

The competitive affinity assay was done using various synthesized *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9**(**a**–**j**) to find out their affinity towards cortical M1 receptor. Male Wistar rat brain cortex was taken out and 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.[18] 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.[19]

The affinity of various compounds towards M1 receptor was 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.[20]; Yamamura and Snyder[21], with slight modification. In brief, an aliquot of synaptosomal membrane proteins (50 μg) was incubated with different concentrations of compounds (0.1–500 μM) as a displacer and [^3H]QNB (0.2 nM), and reaction volume was made up to 200 μl with assay buffer in 96-well plates and incubated for 2 h at 37 °C. The reaction for all displacement assays 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 and added 5 ml of scintillation fluid 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 (McPherson). [22] 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 [^{3}H]myoinositol (s.a. 16.5 Ci/mmol, Amersham) in rat cerebral cortex slices, at basal level and also after stimulation by different concentrations of synthesized *N*-arylthioureas substituted 3-morpholino arecoline **9**(**a**–**j**) derivatives (1–500 μmol/l), 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 (1–100 μmol/l), following the procedure described by Kendall and Naharoski. [23] The radioactivity of [^{3}H]IP3 formed was measured in a liquid scintillation counter at 65% efficiency. The levels of IP3 were expressed as picomoles of [^{3}H]IP3 formed per milligram of protein.

4.2.3. Antiamnesic activity

It was carried out for synthesized *N*-arylthioureas substituted 3-morpholino arecoline derivatives **9**(**a–j**) against scopolamine-induced memory loss using passive avoidance step down task paradigm in male Wistar rats weighing 200–250 g ($n = 8$) according to the method described by Sharma and Kulkarni.[24,25]

4.2.4. Elevated plus maze

This was employed for the measurement of transfer latency (TL). The male Wistar rats (weighing 200–250 g) were placed individually at the end of one arm facing away from the central platform, and the time they took 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 with scopolamine, and scopolamine with test compounds treatment to respective animal groups, 30 min before the plus maze exploration. Control group animals were treated with saline (0.9%). Second day TL was measured in the similar way on the same animals. The resultant data were subjected to statistical analysis. $P < 0.05$ was considered statistically significant.[25]

4.2.5. Y maze task

This task was used to measure the spatial memory through the spontaneous alteration behaviour in rats. Male Wistar rats (weighing 200–250 g) were allowed to explore in Y maze, 30 min prior to this test compounds along with scopolamine were administered to respective animal groups. The ability to alternate requires that the rats know which arm they have already visited. [26] The series of arm entry, including possible returns into the same arm, is recorded visually. Alteration is defined as the number of successive entries into the three arms, on overlapping triplet sets. The percentage of alteration is calculated as the ratios of actual alterations to possible alterations, defined as the total number of arm entries minus two, and multiplied by 100.

4.2.6. Acute toxicity

Rats (8 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 (0.05–0.2 mg/kg) of the derivatives were selected to find optimum dose (found to be 0.1 mg/kg) for in vivo studies.

4.2.8. Data analysis

The data from the radioligand displacement assay were analyzed using 'LIGAND–DRUG' software programme (McPherson)22] to obtain the IC_{50} and K_i values (both are expressed in μmol/l). All the data are expressed as means ± SD. The statistical analysis was done by using one-way analysis of variance (ANOVA). Differences were considered to be significant at $P < 0.05$. All analyses were performed with the 'Jandel-Scientific-Sigma stat' software, version 2.0 for windows.

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