Final Report of the Work Done

Title: Synthesis and biological evaluation of benzoannelated N-rich privileged scaffolds for Neurological disorders

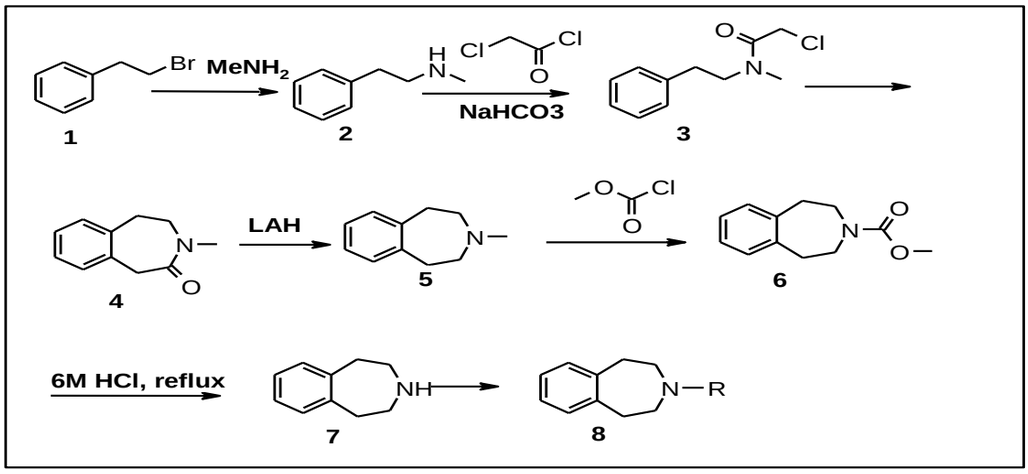
Objectives of the Project

- To develop serotonergic and dopaminergic agents that afford optimum selectivity for the treatment of sexual dysfunction, anxiety disorder, depression and schizophrenia.
- Designing of diversified nitrogen containing new molecular library of compounds with maximum selectivity for D_2, D_3 and $5HT_2$ receptors.
- Synthesis of designed CNS acting agents.
- Well known *N*-containing fused ring system like benzazepines could also be scrutinized for further transformation in order to maximize efficacy and minimize the side effects.
- *In vitro* and *in vivo* biological evaluation of synthesized compounds.

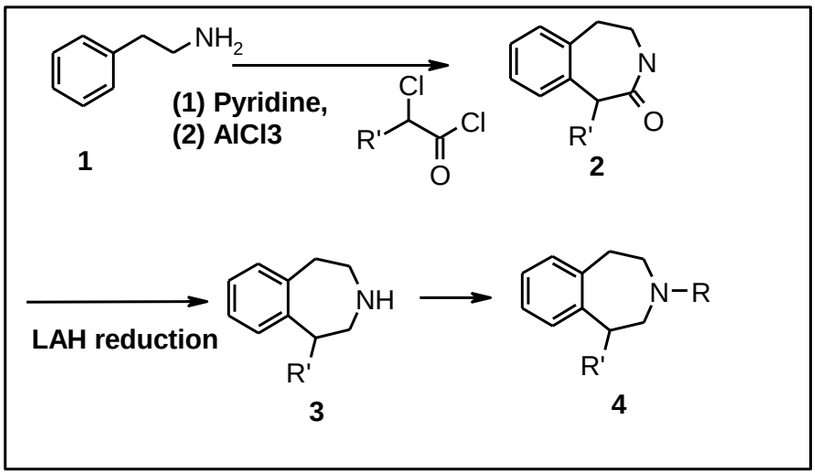
I. Design and Synthesis of Focused library

Synthesis was carried out by using Scheme-I-IV.

Scheme-1



Scheme-2

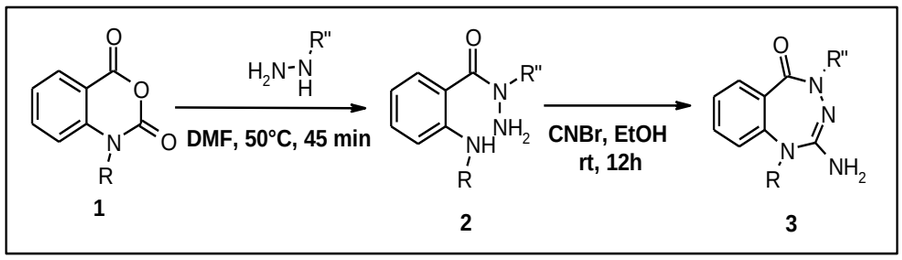


R' = Bn, Et, Me, $(Me)_2$

R = Acid chlorides, Aldehydes, Acids, Sulphonyl chlorides etc.

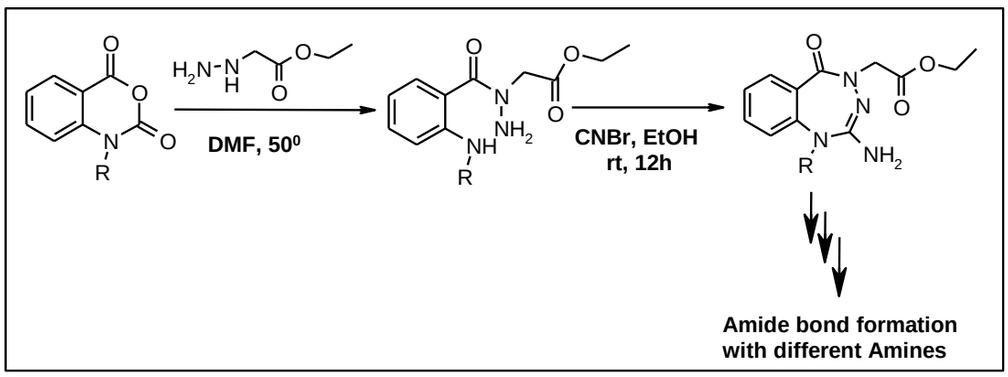
General synthesis of some novel cyclic Guanidines

Scheme-3



R = Me, Bn
R" = Me, Bn

Scheme-4



R = Me, Bn

II. Biological Activity

The synthesized compounds were screened for the following biological activities-

Antipsychotic activity

A. The evaluation of selective dopamine D_2, D_3, 5-HT_6, 5-HT_{2A}, 5-HT_{2c} antagonist activity by radio ligand binding assay.

B. The *in vivo* animal tests to predict the **Antipsychotic activity** was performed as follows-

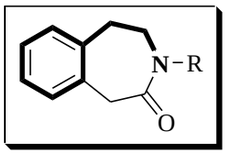
- Behavioral animal model for dopaminergic over-activity used to study the antipsychotic activity of the compounds. Apomorphine and amphetamine induced catalepsy animal model was used to selective blocked of D_2 receptor in mesolimbic and mesostriatal area.
- Conditional Avoidance Response (CAR) in animals were studied by training the animals to perform a certain response to avoid the mild shock (pressing a lever, climbing a pole, jumping out of box).

5-HT modulators

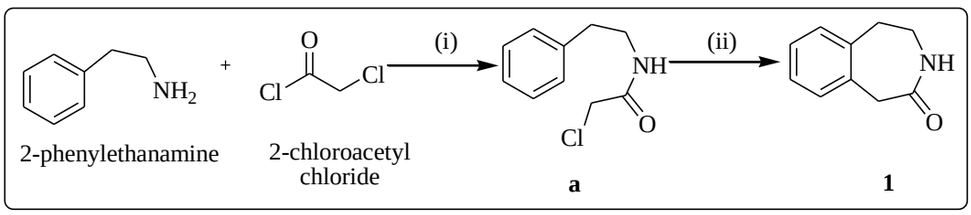
I. Synthesis of Benzoannelated N-rich privileged scaffolds for Neurological disorders

To identify new potentially selective $5HT_{2C}$ agonists we thought of exploring 1- and/or 3-alkylated 3-benzazepines as this ring system contains the phenethylamine fragment present in most of the 5HT mediated CNS acting drugs such as lorcaserin, dexphenfluramine and SB-242084.

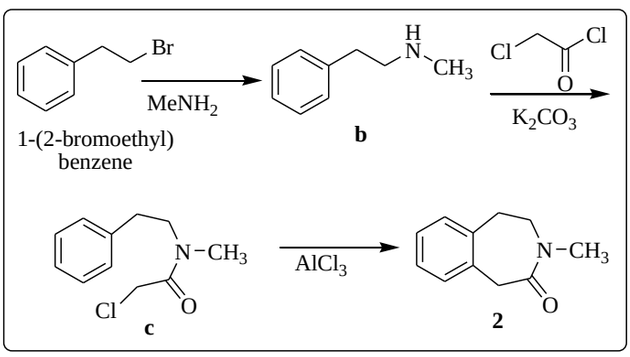
The selectivity aspect for 5-HT_{2C} receptor is important to avoid the side effects caused by interaction of the new moieties with 5-HT_{2A} and 5-HT_{2B} receptors. It was envisaged to synthesize 3-benzazepines substituted at position 1- and/or 3 with various alkyl groups with or without 2-oxo, and biologically evaluate the synthesized compounds for identifying potentially selective $5HT_{2C}$ agonists. It was planned to address the selectivity issue by employing classical pharmacological methods in absence of in house facilities for *in vitro* receptor binding studies. Assuming the synthesis of C- and N-alkylated products to be an easy job it was intended to synthesize a library of compounds using different alkyl groups in the beginning but after facing the purity problems in the synthesis of initial few compounds of the series, syntheses were narrowed down to a smaller number of selected compounds only. Hence, several benzazepine-2-ones were synthesized. The schematic representation is given below. First step was to synthesize derivatives with the structure below using a suitable protocol:



✓ **Synthesis of 4,5-dihydro-1*H*-benzo[*d*]azepin-2(3*H*)-one (1)**

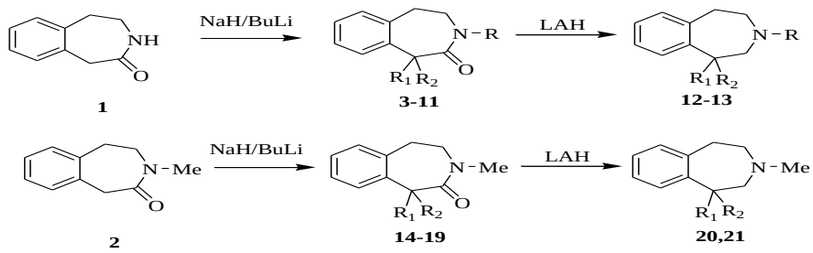


✓ **Synthesis of 3-Methyl-4,5-dihydro-1*H*-benzo[*d*]azepin-2(3*H*)-one (2)**



✓ **Synthesis of target compounds (3-21)**

The synthesized compound underwent substitution at 1 position using strong base like BuLi/NaH and alkyl halide to afford alkylated benzazepine derivative (**3-21**)



Entry	**R**	**R_1**	**R_2**	**Base**	**Yield (%)**
3	Allyl	H	H	BuLi	58.92
4	Benzyl	H	H	BuLi	71.50
5	H	Allyl	H	BuLi	72.86
6	H	Benzyl	H	BuLi	57.29
7	Ethyl	Ethyl	H	BuLi	72.00
8	Benzyl	Benzyl	H	BuLi	86.50
9	Allyl	Allyl	Benzyl	NaH	71.00
10	Benzyl	Benzyl	Benzyl	NaH	62.00
11	Ethyl	Ethyl	H	NaH	80.21
12	Benzyl	Benzyl	H	NaH	81.20
13	Benzyl	Benzyl	Benzyl	NaH	63.20
14	-	Ethyl	H	BuLi	71.60
15	-	Methyl	H	BuLi	76.50
16	-	Allyl	H	BuLi	69.00
17	-	Benzyl	H	BuLi	70.90
18	-	Ethyl	Ethyl	NaH	65.00
19	-	Benzyl	Benzyl	NaH	68.00
20	-	Ethyl	H	NaH	82.75
21	-	Ethyl	Ethyl	NaH	62.00

II. Biological evaluation

✓ *In vitro* isolated rat fundus and isolated rat thoracic aorta experiments

Compounds **2a, 2b, 3a, 3c, 5a, 5b, 9a** and **10a** were found to be active for $5HT_{2B}$ or $5HT_{2A}$ and were eliminated from the study as we require compounds selectively active for $5HT_{2C}$ receptors only. Compounds **3a, 3c, 5a, 5b, 9a** and **10a** have shown agonistic property via $5HT_{2b}$ receptor at 35 µM concentration on rat fundus preparation but were inactive at same concentration on rat thoracic aorta which have shown its inactivity via $5HT_{2a}$ receptor where as compounds **2a** and **2b** have shown antagonist activity on rat thoracic aorta and no activity on rat fundus preparation, which shows the activity of compound **2a** and **2b** on $5HT_{2A}$ receptor. Furthermore compounds **3b, 4a, 4b, 4c, 7a, 7b, 8a, 10b, 11a, 12a, 12b, 12c, 13a** and **14a** has been found inactive on both rat fundus and rat thoracic aorta at 35 µM concentration via $5HT_{2b}$ & $5HT_{2a}$ receptor mechanism.

Table1. Compounds showing effect on isolated rat fundus preparation and rat thoracic aorta preparation at 35 µM concentration.

Comp ID	**Effect of compounds on Isolated rat fundus preparation via $5HT_{2B}$ at 35 µM concentration**			**Effect of compounds on Isolated rat thoracic aorta preparation via $5HT_{2A}$ at 35 µM concentration**		
	Agonist	**Antagonist**	**Inactive**	**Agonist**	**Antagonist**	**Inactive**
3	-	-	+	-	+	-
4	-	-	+	-	+	-
5	+	-	-	-	-	-
7	-	-	+	-	-	+
8	-	-	+	-	-	+
9	-	-	+	-	-	+
10	-	-	+	-	-	+
11	+	-	-	-	-	-
13	-	-	+	-	-	+
16	+	-	-	-	-	-
18	+	-	-	-	-	-
19	-	-	+	-	-	+
21	-	-	+	-	-	+

✓ Tail Suspension Test

In mice, fluoxetine at 10 mg/kg significantly reduces immobility time in the mice TST and used as standard. Initially all the inactive compounds on $5TH_{2B}$ and $5HT_{2A}$ were screened for TST in mice and only potent compounds were further preceded for other *in vivo* evaluation. Compounds **10 and 19** at 10mg/kg i.p. reduced immobility time in the mice tail suspension test (p values <0.05; Fig. 1). But compounds **7, 8, 9, 10, 13 and 19** had not shown significant results. We further evaluated these five compounds for anxiety, hyophagia and penile erection animal models.

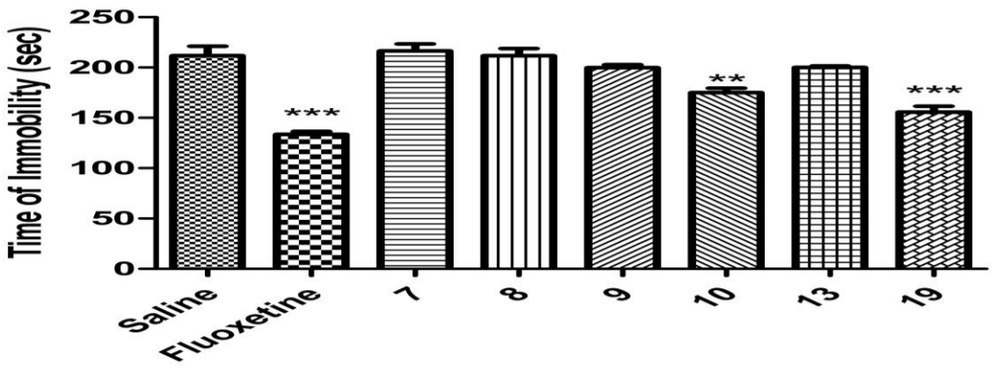


Figure 1- Effect of Fluoxetine, compounds 7, 8, 9, 10, 13 & 19 in immobility produced in the Tail suspension test (TST) in Swiss albino mice. Values represent mean immobility time±SEM. Asterisks indicate values differ from vehicle treatment ($p<0.05$). N=6 per treatment group

- ✓ **Elevated Plus Maze test**

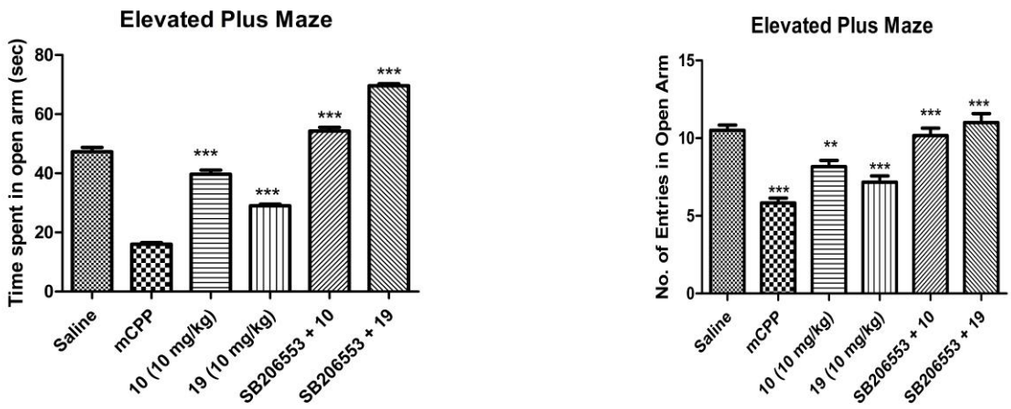


Figure 2.a-Effect of mCPP, 10, 19 and SB-206553 in combination with 10, 19 (i.p. 30 minutes pretest except SB-206553 45 minutes pretest) on time spent in open arms in a mice 5 min Elevated Plus Maze (EPM) test.

Figure 2.b -Effect of mCPP, 10, 19 and SB-206553 in combination with 10, 19 (i.p. 30 minutes pretest except SB-206553 45 minutes pretest) on number of entries in open arms in a mice 5 min Elevated Plus Maze (EPM) test. All data expressed as Mean ± SEM, n = 6. Significantly different from vehicle-treated group: ***P<0.05 by Dunnett's test and 1-way ANOVA.

As expected for a positive control, *m*CPP 1mg/kg i.p., induced a selective anxiogenic-like effect in mice characterized by a significant decrease in the number of open arm entries, without changing the number of enclosed arm entries, compared to negative control, normal saline (Figs. 2.a and 2.b). Treatment with the compounds, **10 and 19** at 10 mg/kg significantly decreased the number of open arm entries and time spent in open arm of mice in the EPM. That demonstrated that **10 and 19** had anxiogenic like activity. Further **10 and 19** at 10mg/kg in presence of SB206553, a selective $5HT_{2C/2B}$ antagonist at 2mg/kg i.p. increased the number of entries in open arm as well as time spent in open arm that showed their selectivity for $5HT_{2C}$ receptors.

- ✓ **Hypophagic Response:** *m*CPP at 5 mg/kg i.p. showed Hypophagic response as expected as compared with control treated animals with normal saline at 10 ml/kg. **10 and 19** also showed Hypophagic responses. However the Hypophagic responses of **10 and 19** were reversed by SB206553 (Figure 3).

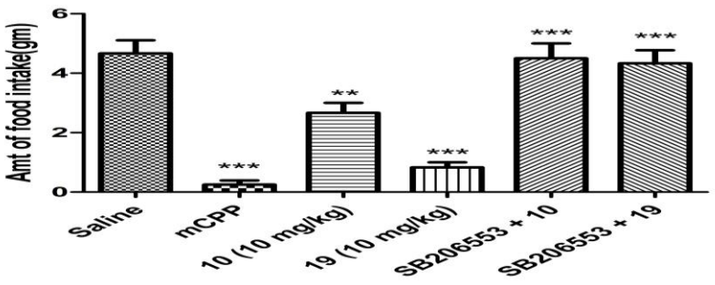


Figure 3 – Effect of SB-206553 on the 10 and 19 mediated reduction in 2-h food intake in 24 h fasted normal Sprague–Dawley rats. Antagonists were administered ip 15 min (SB-206553, 2 mg/kg i.p.) prior to administration of 10 mg/kg, i.p. 10 and 19. *m*CPP as standard was given at 5 mg/kg i.p. Data are expressed as g food consumed in 2-h period and represent mean ± SEM (n = 3 per group). Significantly different from vehicle-treated group: *P<0.05 by Dunnett's test and 1-way ANOVA.

✓ **Penile erections in rats:**

*m*CPP at 0.75 mg/kg s.c.[20] induced penile erections and engorged penis as compared with control treated animals with normal saline at 10 ml/kg. Similarly **10 and 19** had shown some penile erections at dose 20 mg/kg s.c. However penile erections were antagonized by SB206553 at 2 mg/kg (Figure 4).

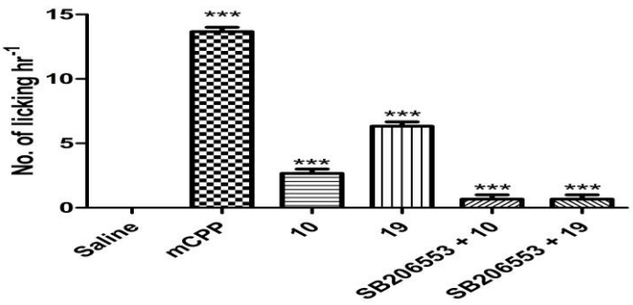
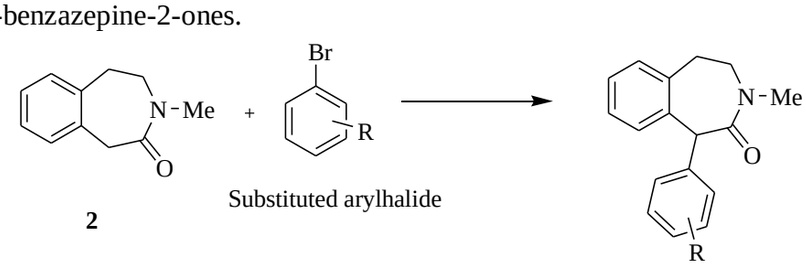


Figure 4- Interaction of SB-206553 with 10 and 19 on penile erections in Sprague Dawley rats. SB-206553 were injected IP 15 min before the IP injection of 10 and 19 (20 mg/kg) and the counting of penile erections during a 60-min period starting from the injection of 10 and 19. Mean± SEM from 6 rats per group. Significantly different from vehicle-treated group: ***P<0.05 by Dunnett's test and 1-way ANOVA**.**

Dopamine Receptor modulators

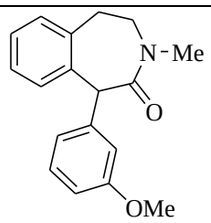
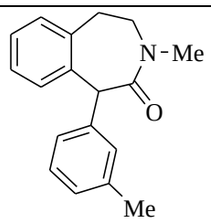
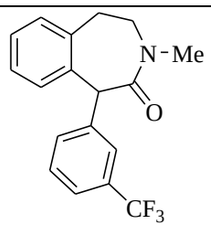
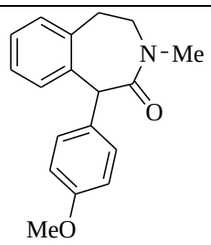
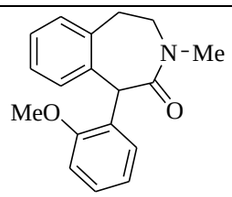
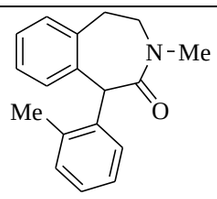
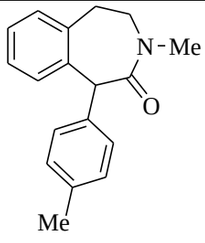
I. Synthesis of some novel 1-Aryl-3-methyl-4,5-dihydro-1H-benzo[d]azepin-2(3*H*)-ones

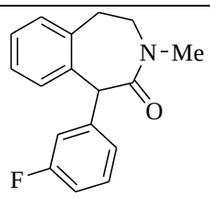
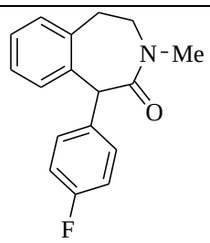
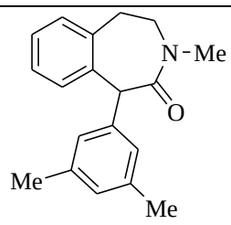
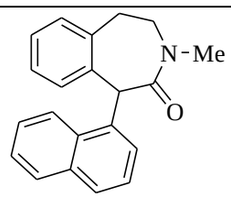
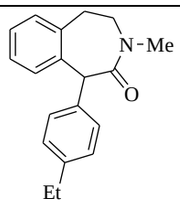
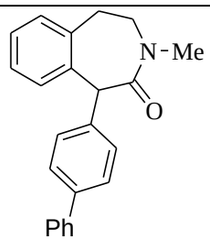
The basic scaffold N-methyl benzazepine **(2)** was subjected to the palladium catalyzed arylation reaction for achieving C-C bond formation in the synthesis of 1-aryl-N-methyl-tetrahydro-benzazepine-2-ones.



2 **22-34**

The details of reaction conditions of the compounds are given in the table below:

Com.	Compound Structure	Base	Reaction Time	Yield (%)
1		BuLi	24 Hrs	35
		NaH	12 Hrs	58
2		BuLi	24 Hrs	31
		NaH	12 Hrs	65
3		BuLi	24 Hrs	25
		NaH	12 Hrs	40
4		BuLi	24 hrs 12 hrs	30 93
5		BuLi	24 Hrs	0
		NaH	12 Hrs	65
6		BuLi	24 Hrs	0
		NaH	12 Hrs	88
7		BuLi	24 Hrs	-
		NaH	12 Hrs	93

8		BuLi	24 Hrs	-
		NaH	12 Hrs	79
9		BuLi	24 Hrs	-
		NaH	12 Hrs	72
10		BuLi	24 Hrs	-
		NaH	12 Hrs	86
11		BuLi	24 Hrs	-
		NaH	12 Hrs	93
12		BuLi	24 Hrs	-
		NaH	12 Hrs	92
13		BuLi	24 Hrs	-
		NaH	12 Hrs	95

Compound showing potential D_1 agonistic activity (4) evaluated for Parkinson's rat model.

RESULTS

Effect of test compounds on rat superior mesenteric artery

As shown in Table 1, the compound **(4)** was found to be D_1 receptor agonists. Compound (**4**) showed selectivity for D_1 receptor as evidenced by their pD_2 values (7.00 ± 0.23, 7.78 ± 0.41 and 7.53 ± 0.57, respectively). Rest of the compounds were excluded from the current study as they were found to be either antagonist or inactive on dopamine (DA) receptors. Compound (**4**) was evaluated further using different *in vitro* and *in vivo* experiments as it showed the highest agonist potency among the series of compounds.

Table 1: Effects of a series of benzazepine derivatives on isolated rat superior mesenteric artery strip.

Com. No.	*p*D_2 value			*p*A_2 value		
	D_1 receptor	D_2 receptor	D_3 receptor	D_1 receptor	D_2 receptor	D_3 receptor
1	--	--	--	5.17±0.12	5.68±0.16	5.72±0.05
2	--	--	--	5.57±0.14	5.60±0.11	5.63±0.13
3	3.72±0.05	3.55±0.13	3.58±0.14	--	--	--
4	**7.00±0.23**	4.28±0.12	3.92±0.17	--	--	--
5	3.98±0.15	4.06±0.16	3.69±0.07	--	--	--
6	--	--	--	5.72±0.14	5.68±0.12	5.74±0.14
7	--	--	--	5.73±0.14	5.67±0.07	5.74±0.18
8	--	--	--	5.64±0.09	5.34±0.10	**7.68±0.15**
9	3.71±0.12	3.71±0.11	2.35±0.09	--	--	--
10	--	--	--	5.48±0.10	5.51±0.14	5.53±0.13
11	4.68±0.07	4.71±0.08	4.73±0.15	--	--	--
12	--	--	--	5.74±0.07	5.83±0.10	5.86±0.14
13	--	--	--	5.71±0.08	5.67±0.16	5.69±0.10

Compound (4) improves 6-OHDA induced injury in human SH-SY5Y neuroblastoma cell culture through D_1 agonism

To determine the toxicity of compound (**4**) on human SH-SY5Y neuroblastoma cells, MTT assay was carried out. Literature suggests that 6-OHDA induced toxicity in human dopaminergic SH-SY5Y cells is a well-established cellular model of PD as these cells efficiently demonstrate the changes in signal transduction pathways associated with the PD progression. Hence, before proceeding for the *in vivo* experiment, the neuroprotective and anti-Parkinson's effect of the compound (**4**) was evaluated using this well established cellular model of PD. In a set of experiments human SH-SY5Y neuroblastoma cells were incubated with 100 µM 6-OHDA for 24 h, with or without different concentrations of compound (**4**) and **A-77636** (5-20 µM). In other set of experiments, cells were incubated with **R-SCH-23390** (10 µM) for 2 h followed by treatment of compound (**4**) or **A-77636** (5-20 µM) for 3 h before 6-OHDA treatment. MTT assay was then performed to assess 6-OHDA induced cytotoxicity, and to assess the neuroprotective role of the test compound (**4**). As shown in Figure 1, 6-OHDA significantly decreased the cell viability. On the other hand, the cytotoxic effects were reduced by pre-treatment of the cells with the compound (**4**) (5-20 µM). The cytotoxic effect of 6-OHDA was significantly blocked by compound (**4**). Furthermore, the protective effect of compound (**4**) was significantly attenuated by R-SCH-23390

(a selective D_1 antagonist). Thus, the results of MTT assay showed that, similar to the standard D_1 agonist **A-77636**, compound (**4**) (5-20 µM) significantly and dose dependently protected human SH-SY5Y neuroblastoma cells against 6-OHDA toxicity through D_1 agonism. These findings were further supported by earlier reports showing cytoprotective effect of DA receptor agonist against 6-OHDA insult.

Behavioral studies

The rotational (circling) behaviour of rodents with unilateral nigrostriatal damage has been broadly utilized to explore nigrostriatal capacity and the activity of dopaminergic compounds. Microinjection of the catecholaminergic neurotoxin, 6-OHDA, was given unilaterally in rats. The lesioned rats impulsively display minor ipsilateral (towards the lesioned side) rotation. Treatment with a DA receptor agonist provokes active contralateral rotation. In accordance to the above notion, compound (**4**) showed significant contralateral rotations similar to the standard D_1 agonist, **A-77636**. However, a behavioural tolerance was observed in case of **A-77636** from fifth day of dosing similar to earlier reports. The results authenticate that compound (**4**) is a selective D_1 agonist.

Antioxidative effects of compound (4) in 6-OHDA-treated rat brain

A previous report demonstrated that thymoquinone (a bioactive constituent of volatile oil of *Nigella sativa* seed) significantly decreased MDA levels and increased SOD levels in 6-OHDA induced Parkinson's rat model at a dose of 5 mg/kg (Sedaghat et al. 2014). Compound (**4**) on the same dose i.e. 5 mg/kg p.o., significantly improvised the oxidative parameters in the 6-OHDA induced Parkinson's rat model. To evaluate whether compound **(4)** exerts antioxidative effect, brain tissues of rats treated with compound **(4)** were subjected to colorimetric estimation to establish its antioxidative properties in the brain. The results showed that 6-OHDA treatment significantly ($p<0.001$) decreased the brain GSH level (1.20±0.12 nM/mg protein) compared to the control group (3.23±0.15 nM/mg proteins). Treatment with the standard drug (**A-77636**) and the test compound (**4**) significantly increased brain GSH levels [**A-77636** (3.07±0.19), and compound (**4)** (2.87±0.19) nM/mg protein] compared to the corresponding 6-OHDA treated group. Further, 6-OHDA treatment significantly ($p<0.001$) increased the brain MDA level (3 .80±0.16 nM/mg protein) compared to the control group (2.20±0.15 nM/mg protein). Treatment with the standard drug (**A-77636**) and the test compound (**4**) significantly decreased brain MDA levels. **A-77636** (2.30±0.15) and compound (**4)** (2.40±0.16) nM/mg protein] compared to the corresponding 6-OHDA treated group .Additionally, 6-OHDA treatment significantly ($p<0.001$) decreased the brain SOD level (29.67±1.20 U/mg protein) compared to the control group (40.67±0.88 U/mg protein). Treatment with the standard drug (**A-77636**) and the test compound (**4**) significantly increased brain SOD levels. **A-77636** (36.67±0.88) and compound (**4)** (37.00± 0.26) U/mg protein] compared to the corresponding 6-OHDA treated group. 6-OHDA treatment also decreased significantly ($p<0.001$) the brain catalase level (2.30±0.16 U/mg protein) compared to the control group (4.00±0.12 U/mg protein). Treatment with the standard drug (**A-77636**) and the test compound (**4**) significantly increased brain catalase levels [**A-77636** (3.80±0.15) and compound (**4)** (3.57±0.19) U/mg protein] compared to the corresponding 6-OHDA treated group. These results clearly revealed antioxidant property of compound (**4)**.

Compound (4) increases dopamine (DA) level in striatum of 6-OHDA lesioned rats

The unilateral 6-OHDA model has the benefit of giving side-biased motor impairments. The 6-OHDA lesion model can be used in both rats and mice. Unilateral 6-OHDA model is the traditional model for assessing Parkinson's treatments, particularly those proposed to enhance DA levels in the striatum. The main positive point of this model is that it is particularly sensitive to DA receptor agonists. The toxin 6-OHDA is injected unilaterally, while the inverse half serves as an intra-animal control. This injection produces loss of dopaminergic neuron on the 6-OHDA-injected side while saving the contralateral dopaminergic neurons. In line to the above note, 6-OHDA treatment

significantly ($p<0.001$) decreased the striatum DA level (2.04±0.15 µg/gm tissue) compared to the control group (5.94±0.15 µg/gm tissue). Treatment with the standard drug (**A-77636**) and the test compounds (**4**) at 0.1 mg/ kg and 5 mg/kg respectively, significantly increased striatum DA levels (**Figure 1**) [**A-77636** (5.46±0.16) and compound (**4)** (5.30±0.15) µg/gm tissue] compared to the corresponding 6-OHDA treated group (**Figure 1**). Previously, Chan et al. (Chan et al. 2013) reported that DBZIM (an imidazolium compound) at 6 mg/kg significantly maintained brain DA level in 6-OHDA induced Parkinson's rat model. In the present study, compound (**4**) at 5 mg/kg significantly increased striatum DA levels. These results demonstrated the anti-Parkinsonian effect of compound (**4)**.

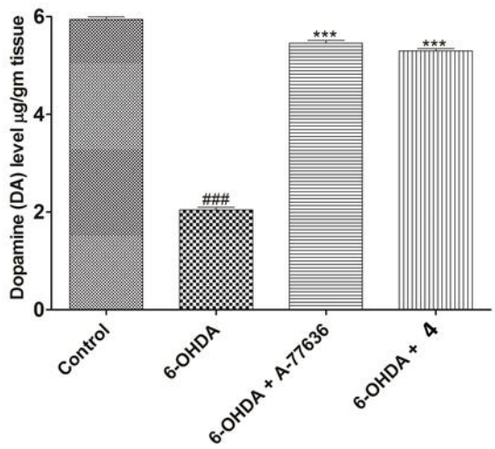


Figure 1: Effect of compound (4) on dopamine (DA) level *ex vivo* in 6-OHDA induced Parkinson's rat model.

Compound (4) diminishes cleaved caspase-3 and increased tyrosine hydroxylase (TH) expression in 6-OHDA-induced Parkinson's rat brain

In the current study, the expression of TH and cleaved caspase-3 were determined to confirm the neuroprotective property of the compound (**4**).

Discussion

The DA D_1 receptor is confined in high concentrations in some of the anatomically different parts of the rodent, monkey and human basal ganglia. A far reaching assemblage of behavioral and biochemical confirmations shows a contribution of the D_1 receptor in the functioning of the rat basal ganglia. In any case, the role(s) of D_1 receptors in the functioning of the monkey and human basal ganglia stays dark. SKF 38393, the most broadly utilized D_1 agonist, remains without remedial effect for the Parkinsonian manifestations of either MPTP-treated primates or people with idiopathic Parkinsonism. A series of isochromans having potent, specific and long-acting D_1 receptor agonistic activities have been accounted biologically. **A-77636** ((1*R*, 3*S*) 3-(l'-adamantyl)-1-aminomethyl-3,4-dihydro-5,6-dihydroxy-l*H*-2-benzopyran hydrochloride) is an alternate D_1 receptor-specific isochroman with *in vitro* and *in vivo* D_1 agonistic activity. Previous studies demonstrated that **A-77636** eases the Parkinsonian manifestations of marmosets treated with the neurotoxin MPTP. These observations and some others display that D_1 receptor plays an important role in the working of the extrapyramidal nervous system of primates.

Participation of D_l receptors in the performance of the extrapyramidal nervous system is in agreement with many observations; high concentrations of D_1 receptors exist in several sites inside the basal ganglia, most notably the substantia nigra pars reticulata and caudate putamen; the formation of a second messenger (cyclic-AMP) was stimulated by D_1 agonists in the tissues either from the substantia nigra or the caudate-putamen.

Similarly, on emulating damage to the striato-nigral dopaminergic neurons with 6-OHDA, D_1 receptors (not D_2 receptor) get stimulated causing increased utilization of glucose in the substantia

nigra pars reticulata and the entopeduncular nucleus. *L*-DOPA, the most widely utilized therapeutic agent for PD, likewise increases glucose utilization in this experimental model. D_1 agonist **A68930**, an isochroman, was demonstrated to additionally increase nigral growth of 2-deoxyglucose. These observations clearly demonstrated that D_1 receptor plays an important role in the physiology of the extrapyramidal nervous system. Thus, the D_1 receptor is in focus for rational designing of anti-Parkinsonism agents.

A series of benzazepines were synthesized to assess their potential as D_1 agonists. The synthesized compounds were evaluated for their D_1 agonistic potential using isolated mesenteric artery preparation. Compound (**4**) emerged prominently as a potent D_1 agonist in these experiments. A brief structure activity relationship study revealed that aryl group at C-1 position of the 3-benzazepine ring system is conductive for activity on dopaminergic receptors. DA receptor antagonist activity is more prominent than the agonist activity for majority of the substituents except for compound (**4**) which showed a potent agonistic activity for D_1 receptor. Methyl group at all the three positions in the phenyl ring offers compounds having antagonistic activity on all the subtypes of DA receptors. Same is the fate of ethyl and phenyl groups providing antagonist compounds. Methoxy group at *ortho* and *para* (**4**) positions of phenyl ring offered agonists with the maximum activity residing in the *p*-methoxy derivative (**4**) for D_1 receptor while the *meta* derivative was observed to show antagonism on all the three receptor subtypes. *m*-Fluoro derivative offered antagonism on all the three receptors with the maximum activity shown for D_3 subtype. *m*-Trifluoromethyl group offered mild agonistic activity for all the three subtypes. It could be concluded that powerful electron donating or accepting groups at *para* position of the phenyl ring offers DA receptor agonists while rest of the groups at other positions offer compounds having DA receptor antagonistic activity. Since human SH-SY5Y cell lines insulted by 6-OHDA is a well-established cellular model of PD, the compound (**4**) was further assessed against 6-OHDA-induced injury in human SH-SY5Y neuroblastoma cell lines. The compound (**4**) showed neuroprotection in the cultured cells through D_1 agonism similar to the standard **A-77636**. Earlier findings further support the cytoprotective effect of DA receptor agonist.

According to previous reports, dinapsoline (DNS)-treated 6-OHDA-induced unilateral lesioned rats in the medial forebrain bundle showed vigorous contralateral turnings. The DNS-induced rotational behaviour emerges to be the case of *in vivo* D_1 receptor stimulation. The rotations induced by DNS were fully blocked by D_1-selective antagonist SCH-23390 while no effect on rotations was shown by D_2-selective antagonist raclopride. In the experiments used for the rat unilateral 6-OHDA rotation model of PD, 6-OHDA is injected unilaterally into the substantia nigra, or the striatum and the medial forebrain bundle. These results in the damage of dopaminergic terminals and neurons with loss of striatal DA, as a result reflective functional dopaminergic supersensitivity develops on the lesioned side. When treated with direct-acting DA receptor agonists, unilaterally 6-OHDA lesioned rats turn contralaterally (far from the side of injury) on account of the increased sensitivity of the postsynaptic DA receptors on the lesioned side. Compound (**4**), which was found to be a proven D_1 agonist in the preliminary *in vitro* studies, was further evaluated using 6-OHDA unilaterally lesioned Parkinson's rat model. Compound (**4**) significantly increased the number of contralateral rotations in 6-OHDA induced unilateral lesioned rats. This showed that compound (**4**) is having affinity for D_1 receptors and the rotations were due to D_1 receptor stimulation.

6-OHDA is a neurotoxin that offers functional animal model of PD by inducing the degeneration of DA neurons in the SNpc and injures the nerve endings in the striatum. According to previous reports, 6-OHDA lesioning leads to degeneration of dopaminergic neurons which results in significant neurochemical changes such as a decrease in DA content and reduced TH immunoreactivity. In the present study, 6-OHDA significantly decreased ipsilateral striatal DA level and reduced TH expression in ipsilateral SNpc region which indicate degeneration of dopaminergic neurons. Treatment with the compound (**4)** significantly increased the DA level and the expression of TH in 6-OHDA-induced Parkinson's rat brain. This showed that compound (**4**)

possessed neuroprotective property against 6-OHDA induced PD in rat.

Apoptosis is likewise a pigeonholed biological procedure through which cells are disposed off during cell development because of injury. Under numerous neurodegenerative diseases, apoptosis has been found to cause neuronal cell death. PD may be likewise categorized by progressive degeneration of dopaminergic neurons by apoptosis of the nigrostriatal organization. Furthermore, mitochondrial or Fas- pathways mediate caspase-3 activation induced by 6-OHDA in cells . Even though various mechanisms have been involved in the progression of PD, a thought has been made that oxidative stress also plays some role in the progression of neurodegeneration in PD.

Detoxification pathways have been recognized in various cells to control the hazardous effects of oxidative stress. Of the different antioxidants in the brain, the GSH framework is especially critical in controlling cell redox state and is an essential protection pathway for peroxide expulsion from the brain. In addition, the MDA levels may be dominatingly reliant on ROS levels, including hydroxyl radicals. Hence, MDA (a non-radical product), which is cytotoxic in itself from LPO, may play an essential role in the altered progression induced by unilateral i.c.v. injection of 6-OHDA. After getting significant results in behavioural studies, the compound (**4**) was further evaluated for its potential antioxidative effects. The compound (**4**) significantly and positively modified the oxidative parameters viz. GSH, MDA, SOD and catalase. Treatment with the compound (**4**) significantly increased GSH, SOD and catalase levels while significantly decreased MDA level in 6-OHDA induced Parkinson's rat brain. These results proved that compound (**4**) also possessed antioxidant property.

One of the most important apoptotic activators is the caspase family of enzymes. As a rule, caspase-3 is known to play an essential role in the final regulated pathway of apoptosis. Fas (one of the death receptors present on the surface of the cell) corresponds to a pathway practically controlled by caspases solely. According to this pathway, binding of a ligand to the death receptor leads to accumulation of a series of proteins, which leads to activation of pro-caspase-8. The following proceedings are the strongest indication that caspases work in a cascade manner, with caspase-8 activating caspase-3, which further activates other caspases that cleave different substrates. Caspase-3 sets free caspase-dependent endonuclease (one of the substrates) from its inhibitor in the cytoplasm which then consequently penetrates into the nucleus, where it slices DNA into oligonucleosomal fragments. In an alternate pathway of apoptosis, mitochondrial dysfunction arises during apoptosis leading to the release of cytochrome c from the mitochondria into cytosol, where it attaches to apoptotic protease activating factor 1 (Apaf-1) that restrains binding locates for cytochrome c. The complex then initiates the apoptosome, selects and ties pro-caspase-9 by Apaf-1. Further, caspase-3 has been activated by mature caspase-9 released from the multimeric complex. Therefore, 6-OHDA provokes caspase-3 activation mediated by an after effect of either the mitochondrial- or Fas-pathways. In agreement with the above reports, it was planned to evaluate whether compound (**4**) has any effect on caspase-3 levels. For this purpose, the animals were sacrificed and the brain sections were utilized for immunohistochemical analysis for cleaved caspase-3. Confocal microscopy images demonstrated that 6-OHDA significantly increased cleaved caspase-3 expression in substantia nigra region. Compound (**4**) significantly decreased cleaved caspase-3 expression in substantia nigra region of 6-OHDA induced Parkinson's rat brain. This validated that compound (**4**) demonstrated neuroprotective activity through D_1 agonism and the anti-oxidant mechanism may be responsible in part for its neuroprotective effect. However, further studies are still needed to explore molecular mechanisms involved in anti-Parkinson's effect of compound (**4**). Since there are very few D_1 agonist medications available to treat PD, compound (**4**) could be a potential lead for development of a drug candidate against PD.

RESULTS

D_3 Antagonist activity of test compounds on rat superior mesenteric artery strip

All the test compounds were evaluated for their DA receptor modulator activity as reported earlier. Amongst which compound (**8**) showed potent D_3 antagonist activity while compound (**4**) showed preferential D_1 agonist activity. Compound **(8)** had no vasoconstrictor effect on arterial vessels on their own. **8** significantly attenuated 8-OH-PBZI mediated relaxation with a pA_2 values of 7.68±0.15, 8.12 ± 0.34 and 7.48 ± 0.37 respectively (Table 1, D1 agonist study). Compound (**8**) showed the least effect on A-77636 and bromocriptine mediated relaxation which was evidenced by their pA_2 values. These results suggest that compound (**8**) is potent and relatively selective D_3 antagonist which could be effective to treat neuropsychiatric conditions. Among the potent D_3 receptor antagonists, compounds (**8**) were chosen for further evaluation for antipsychotic activity using different *in vivo* experimental rodent models.

Compound (8) attenuated apomorphine-induced stereotype behaviour

Apomorphine, a non-specific DA receptor agonist, produces stereotype behavior. As shown in Fig. 1A, pre-treatment of the animals with compound (**8**) (5, 10 and 20 mg/kg, p.o.) significantly abrogated apomorphine-induced stereotype behavior (p<0.001). As expected, clozapine (5 and 10 mg/kg, p.o.) also significantly attenuated apomorphine-induced stereotype behavior. The results supported DA receptor antagonist activity of compounds (**8** and **15**).

Compound (8) attenuated spontaneous locomotor activity

Animals treated with compound (**8**) (5, 10 and 20 mg/kg, p.o.) showed significantly reduced spontaneous locomotor activity as compared to the vehicle-treated control group (Fig. 2B, p<0.001). Clozapine (5 and 10 mg/kg, p.o.) as a standard drug also significantly attenuated spontaneous locomotor activity (Fig. 2B, p<0.001) as compared to the vehicle-treated control group. These results point out the antipsychotic potential of compound (**8**).

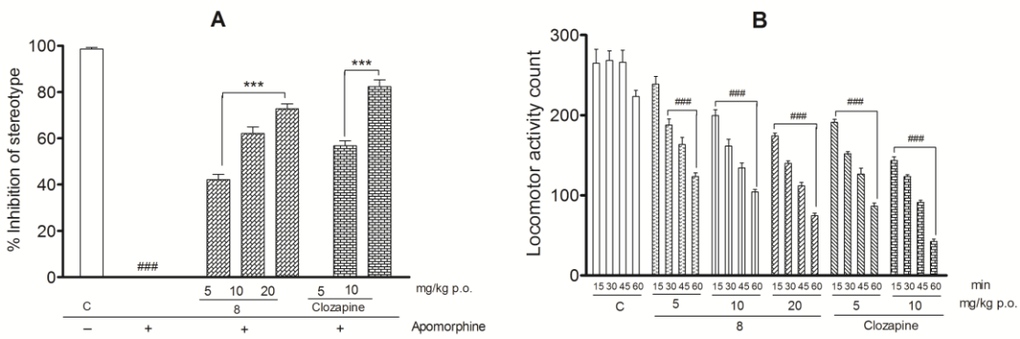


Fig. 2: Compound (**8**) attenuated apomorphine-induced stereotype behaviour (A) and spontaneous locomotor activity (B). ### p<0.001, when compared to the vehicle-treated control group. *** p<0.001, when compared to the apomorphine-treated control group (n=6).

Compound (8) attenuated 7-OH-DPAT-induced hypothermia

8-OH-DPAT, a selective D_3 agonist, elicited significant hypothermia (Fig. 3A, p<0.001) as compared to the vehicle-treated control group. Pre-treatment of the animals with compound (**8**) significantly attenuated 8-OH-DPAT-induced hypothermia in a dose dependant manner (Fig. 2A, p<0.01). **8** did not produce hypothermia on its own. In contrast to this, clozapine (5 and 10mg/kg) by itself elicited significant hypothermia (Fig. 3A, p<0.01). Hence, treatment of clozapine potentiated 6-OH-DPAT-induced hypothermia (Fig. 2A, p<0.001). The results further supported selective D_3 antagonist activity of **8**.

Compound (8) did not induce catalepsy at low doses

In neuropsychiatric conditions, the major drawback of targeting D_2 receptor is the development of motor alteration because of extrapyramidal side effects. As shown in Fig. 2B, compound (**8**)-treated animals did not show any catalepsy response at moderate doses (5 and 10 mg/kg, p.o.) while at a relatively higher dose (20 mg/kg, p.o.), **8** induced significant level of catalepsy (Fig. 2B, p<0.001) as compared to the vehicle-treated control animals. Clozapine (5 and 10 mg/kg, p.o.), as expected, induced significant catalepsy (Fig. 3B, p<0.001), as compared to the vehicle-treated control group even at moderate doses (5 and 10 mg/kg, p.o.) which lasted up to 120 min.

Compound (8) did not induce rota rod ataxia

Rota rod ataxia is another useful tool to evaluate motor alteration in test animals. Compound (**8**), at moderate doses (5 and 10 mg/kg, p.o.), did not induce ataxia in rota rod test after 1 hr of post treatment period (Fig. 2C). However, at a relatively higher dose, **8** (20 mg/kg, p.o.) induced significant ataxia impairing rota rod performance as compared to the vehicle-treated control group (Fig. 2C, $p<0.001$). In contrast to this, clozapine induced significant alteration of rota rod performance in the animals even at lower doses (5 and 10 mg/kg) as compared to the vehicle-treated control animals (Fig. 2C, $p<0.001$). The results suggest that **8** has relatively lesser propensity to induce extrapyramidal side effects as compared to clozapine.

Compound (8) attenuated apomorphine-induced striatal DA level

Administration of apomorphine significantly elevated the striatal DA level in rat brains as compared to the vehicle-treated control group (Fig. 3D, $p<0.001$). Compound (**8**) showed promising antipsychotic effects in different behavioural rodent models. In support of this finding, **8** significantly attenuated apomorphine-induced striatal DA levels in a dose dependant manner (Fig. 3D, $p<0.01$). The results further confirmed DA receptor antagonist potential of **8**.

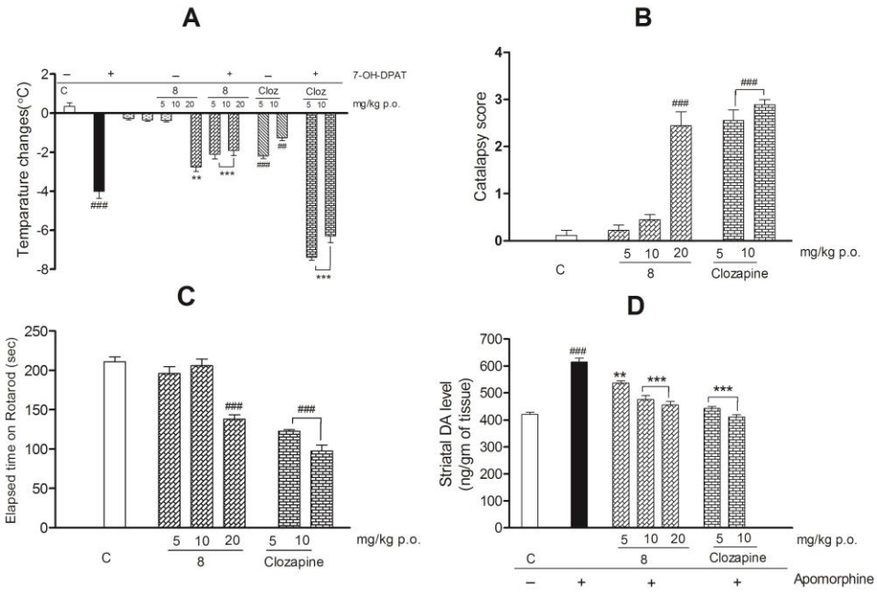


Fig. 3: Compound (**8**) attenuated 7-OH-DPAT-induced hypothermia. In contrast to this, Clozapine potentiated 7-OH-DPAT-induced hypothermia (A). **8** did not induce catalepsy (B) or rota rod ataxia (C) at moderate doses. In difference, clozapine induced significant catalepsy (B) and rota rod ataxia (C) at the same dose levels. **8** attenuated apomorphine-induced striatal dopamine (DA) levels in rat brain (D). ## $p<0.01$, ### $p<0.001$, when compared to the vehicle-treated control group. ** $p<0.01$, *** $p<0.001$, when compared to the 7-OH-DPAT/apomorphine-treated control group (n=6).

Compound (8) were found safe in acute lethality test

Treatment of compound (**8**) up to 300 mg/kg, p.o. did not cause any mortality in the animals up to 14 days ($LD_{50}\geq300$ mg/kg, p.o.). In contrast, all the mice treated with clozapine (100 mg/kg, p.o.) died within 48 hr of drug treatment. Thus, LD_{50} value of clozapine was found to be less than 100 mg/kg, p.o. while **8** was found to be safe up to 300 mg/kg, p.o. dose.

DISCUSSION

Earlier reports have demonstrated selective D_3 receptor antagonist potential of some benzazepine derivatives. Antipsychotic potential of benzazepine derivatives has also been revealed in different rodent models of psychosis which further supported their suitability as a therapeutic alternative in neuropsychiatric diseases. In the present study, DA receptor modulating properties of a series of benzazepine derivatives have been evaluated *in vitro* using isolated rat superior mesenteric artery strips. Amongst the compounds so evaluated, **8** was found to be the most potent and relatively more selective D_3 antagonists. Compound **(8)** significantly attenuated 7-OH-PBZI (selective D_3 agonist) mediated relaxation of pre-constricted mesenteric arterial strips. Compound **(8)**-mediated

D_3 antagonist activity was evidenced by their pA_2 values of 7.68±0.15 and 8.12±0.34 respectively. Compound (**8)** has shown least effect on A-77636 (selective D_1 agonist) or bromocriptine (preferential D_2 agonist) mediated relaxation suggesting their relatively selective D_3 antagonist activity. After determining D_3 receptor antagonist activity in the *in vitro* evaluation, compound **(8)** was further assessed in different behavioural rodent models for antipsychotic activity. Apomorphine (0.5 mg/kg, s.c.) induced significant stereotype behavior in the treated animals as compared to the vehicle-treated control animals. However, compound (**8)** attenuated apomorphine-induced stereotype behavior in rats. Apomorphine-induced stereotype behaviour is attributed to the stimulation of striatal DA receptors and therefore this model is used for screening of DA receptor agonists and/or antagonists to determine their striatal DA receptor activity. The ability of compound **(8)** to attenuate apomorphine-induced stereotype behavior suggests their DA receptor antagonist activity.

Antagonism of DA receptor mediated locomotor activity is a useful tool to determine antipsychotic potential of a compound . In this study, compound **(8)** significantly attenuated spontaneous locomotor activity as compared to the vehicle-treated control group which indicated antipsychotic effect of **8**. Supporting previous reports, Clozapine, a D_4 receptor antagonist, also attenuated spontaneous locomotion in rodents. Some reports also state that preferential D_3 antagonists do not block DA receptor mediated locomotion, rather they promote hyperlocomotor activity. However, selective D_3 antagonists such as S18126 and PD152255 have exhibited significant attenuation of the DA receptor mediated locomotor activity. The reason behind this discrepancy might be attributed to the difference in receptor selectivity and/or procedural differences. Thus, the precise role of D_3 receptor agonist/antagonist in locomotion is still a subject of investigation using more potent and selective ligands acting as D_3 agonist/antagonist.

8-OH-DPAT mediated hypothermia is attributed to the activation of postsynaptic D_3 receptors. Hence, to evaluate the postsynaptic D_3 receptor antagonist activity of the test compounds, 8-OH-DPAT-induced hypothermia rodent model was used. 8-OH-DPAT (0.2 mg/kg, s.c.), a selective D_3 agonist, induced significant hypothermia in rats as compared to the vehicle-treated control animals. However, pre-treatment of the animals with compound **(8)** significantly attenuated 8-OH-DPAT-induced hypothermia in a dose dependant manner. There are evidences which have demonstrated attenuation of 8-OH-DPAT-induced hypothermia using selective D_3 antagonists, which further supported the present finding. In contrast to this, clozapine has distinct hypothermic effect. Treatment with clozapine elicited significant hypothermia as compared to the vehicle-treated control animals. Thus, clozapine potentiated 7-OH-DPAT-induced hypothermia. Clozapine is a potent D_4 antagonist which potentiates 8-OH-DPAT-induced hypothermia instead of attenuating it, suggesting that D_4 receptor blockade is inadequate to abrogate 8-OH-DPAT-induced hypothermia. The results further revealed selectivity in D_3 antagonist activity of **8**, as they significantly attenuated selective D_3 agonist induced hypothermia.

It has been reported that blockade of striatal D_2 receptors leads to induction of extrapyramidal side effects such as catalepsy and rota rod ataxia. However, preferential D_3 or D_4 receptor antagonists are least prone to induce extrapyramidal side effects. Therefore, to rule out D_2 antagonist activity for the test compounds, the ability of the compound **(8)** to induce catalepsy and rota rod ataxia was assessed. It was found that **8** and **15** did not induce catalepsy or rota rod ataxia at moderate doses (5 and 10 mg/kg, p.o.). However, at the same doses, Compound (**8)** has significantly attenuated apomorphine-induced stereotype behavior. At a relatively higher dose (20 mg/kg, p.o.) the test compound **(8)** induced significant catalepsy as well as rota rod ataxia compared to the vehicle-treated control animals which might be attributed to their D_2 antagonist activity at higher concentrations. In contrast to this, clozapine significantly induced catalepsy and rota rod ataxia even at lower doses (5 and 10 mg/kg, p.o.). Thus, the results revealed that compound **(8)** is devoid of D_2 antagonist activity at relatively lower doses which further point out towards the antipsychotic potential of the compound **(8)** with low incidence of extrapyramidal side effects.

Finally, the DA receptor antagonist activity of **8** was assessed by determining striatal DA levels

following apomorphine-induced stereotype behaviour rat model. Apomorphine administration significantly elevated striatal DA levels in rat brains as compared to the vehicle-treated control group. However, pre-treatment of the animals with compound **(8)** significantly attenuated apomorphine-induced striatal DA levels. The ability of **8** to normalize striatal DA levels is attributed to their DA receptor antagonist activity. Previous reports have demonstrated that selective D_3 antagonists normalized elevated brain DA level which further supported the present finding. Moreover, compound **(8)** did not produce any observable behavioural side effects and were relatively safe in acute lethality test in mice (LD_{50}≥300mg/kg, p.o.).

Most of the currently available antipsychotic drugs target D_2 receptors. However, selective blockade of D_2 receptor develops extrapyramidal side effects which are less common with preferential D_3 antagonists. The beneficial effects of D_3 blockade is mainly attributed to the atypical distribution of D_3 receptors in the brain. As compared to other D_2 family members, the D_3 receptors are few in numbers but have predominant abundance in the brain regions which are considered as the centres for emotional, memory and motor functions. Thus, selective D_3 antagonists could be useful to treat neuropsychiatric conditions without producing extrapyramidal side effects. In the present study, novel benzazepine derivative (**8)** was found to be potent and preferentially selective D_3 receptor antagonists that have significant antipsychotic activity with low incidence of extrapyramidal side effects. However, further studies are needed to determine the selectivity of compound **(8)** towards several other receptor subtypes for their complete pharmacological profiling.

List of the Publications resulting from the work

- D3 Antagonist and Antipsychotic Potential of Some Novel Benzazepines. Journal of Pharmaceutical Sciences and Pharmacology, 2015, 2(2), pp.123-133.
- 3-Substituted 1-methyl-3-benzazepin-2-ones as 5-HT 2C receptor agonists. RSC Advances, 2015, 5(111), pp.91908-91921.

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