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Anxiolytic Effect of a Novel 5-HT₃ Receptor Antagonist (4-Phenylpiperazin-1-yl) (Quinoxalin-2-yl) Methanone (4a) in a Battery of Behavioral Models for Anxiety in Mice.

Yeshwant Vijay Kurhe [1*], Mahesh Radhakrishnan [1], Deepali Gupta [1], Devodoss Thangaraj [1]

1. Department of Pharmacy, Birla Institute of Technology and Science, Pilani, Rajasthan-333031, India.
   [E-mail: yashkurhe@gmail.com; Tel: +91-8094693209]

The serotonergic system of brain is well understood to play a crucial role in emotional processing in human and animals. 5-HT₃ receptor, the ion channel type of receptor is involved in mood, anxiety, depression, learning and memory. Objective of the present work was to investigate the anxiolytic potential of a novel 5-HT₃ receptor antagonist (4-phenylpiperazin-1-yl) (quinoxalin-2-yl) methanone (4a) in mice. Different behavioral test paradigms for anxiety like elevated plus maze, open field test, light-dark model and hole board test were used for assessing the effect. Diazepam 2 mg/kg i.p. served as a reference standard. From the results it was found that 4a dose dependently at 2 and 4 mg/kg i.p. attenuated the behavioral alterations in the anxiety models in mice. The exact mechanism of 5-HT₃ receptor antagonist for anxiolytic potential is still ill understood. Our further studies will deal with mechanisms at molecular levels for anxiolytic potential of 4a.

Keyword: 5-HT₃ Receptor, Anxiety, Elevated Plus Maze, Open Field Test, 4a.

1. Introduction

Anxiety is a psychological behavioral disorder which drastically sticks to the mood, emotion and fear. Till today, benzodiazepines (BZD) serve as a choice of drug for anxiety which replaced barbiturates because of their safety and effectiveness [1-2]. But, prolonged use of BZD leads to some serious side effects like, tolerance, physical and psychological dependence, withdrawal symptoms and ataxia [3]. Further, it was thought that the anxiolytic agents exerting is effect through neuronal system and not directly binding to BZD receptors could provide a better therapeutic agents for anxiety disorder. One of the neurotransmitter, serotonin has been studied with this concern for a long time.

The role of serotonin in anxiety profile has been studied previously and it is well accepted that serotonin plays a crucial role in anxiety disorder [4-6]. 5-HT₃ serotonergic receptors because of its selective ligands, is identified and characterized in the brain tissues [7-8]. The physiological role of 5-HT₃ receptors and its ligands as a therapeutics for the mental disorder has not been yet understood clearly. Two decades early, some useful work was initiated for 5-HT₃ receptor antagonist as anti-anxiety agents. But, due to selectivity issues, the work in this area was not proved at mechanistic level. 5-HT₃ receptors antagonists like ondansetron, granisetron, zacopride and tropisetron has been reported for some promising results in animal models of anxiety [9-10].

Clinical reports of 5-HT$_3$ antagonist have shown some promising results. Preliminary clinical studies of ondansetron are being reported for reducing the symptoms of obsessive-compulsive disorder (OCD) [11]. These promising reports are of quiet importance as the classical BDZ are mostly ineffective in treating this disorder [12-13]. Another 5-HT$_3$ receptor antagonist tropisetron was found to be effective in the treatment of generalized anxiety disorder [14]. Further, literature survey reveals that ondansetron reduces the intensity of panic attacks [15] and panic symptoms in panic disorder patients [16]. However, some contraindicatory reports on ondansetron for panic disorder have also reported earlier [17] and as yet, no 5-HT$_3$ receptor antagonist are marketed for treatment of any anxiety disorder [18].

On the basis of above literature we designed and synthesised the 5-HT$_3$ receptor antagonist (4-phenylpiperazin-1-yl) (quinoxalin-2-yl) methanone (4a) with an optimal log P (2.84) and pA$_2$ value (7.3), greater than ondansetron (6.9) was chosen for screening in a chronic model of depression. Our previous finding have dealt with the screening of 4a in acute models of depression [19]. The present study was designed to evalute the anxiolytic potential in mice by using the highly valid models for anxiety like, elevated plus maze (EPM), open field test (OFT), light-dark model (LD), hole board test (HBT).

2. Materials and Methods
2.1 Animals:
Swiss albino mice (male:female 1:1) (20-25 g) were procured from Hisar Agricultural University, Hisar, India (Reg. No. 417/01/a/ CPCSEA). The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. Each group consisted of 6 animals. All the experiments were carried out between 0900 and 1500h. The experimental protocols were approved by Institutional Animal Ethics Committee (IAEC) of Birla Institute of Technology and Science, Pilani, India (Protocol No. IAEC/RES/16/06).

2.2 Drugs and Chemicals:
4a was synthesized by medicinal chemistry group of the Institute. Ampoules of diazepam were procured from BITS Medical Services. Vehicle used for formulation was distilled water. The test compound 4a as well as standard diazepam were prepared freshly in distilled water on the day of experiment and injected in intra-peritoneal cavity (i.p.) at a dose volume of 10 ml/kg for mice.

2.3 Chemistry of 4a:
Chemistry of compound 4a (fig.1) is reported in our earlier publications [19,20].

2.4 Behavioral assays:
2.4.1 Elevated plus maze (EPM):
The elevated plus maze was similar to the one described by Rodgers et al. [21]. Briefly, it consisted of two open and two closed arms (all arms: 20x4x12 cm) made of wooden blocks elevated at a height of 25 cm from floor, which was lighten with 60 watt bulb through a height of 100cm. Each mouse was placed in the central square (5 cm×5 cm) facing an open arm and allowed to explore the maze for 5min of test period. The parameter measured was the time spent and number of entries in open arm. The maze was cleaned with dilute alcohol in between two test sessions to get rid of residual odor.

2.4.2 Open Field Test (OFT):
The open field exploration was performed as described previously by Redmond et al. [22]. The apparatus consisted of a circular (90 cm diameter) arena with 75 cm high aluminum walls and white floor equally divided into 10 cm squares. A 60 W light bulb was positioned 90 cm above the base of the arena, which was the only source of illumination in the testing room. During the test, each animal was individually placed in the center of the open field apparatus and the following parameters were recorded for 5 min by trained observer unaware of the specific treatments, number of central and peripheral ambulation score, number of rearing episodes and fecal pellets. Each mouse was transported, one hour before to the testing room using the home cage. After each test, the

apparatus was sprayed with dilute alcohol and wiped thoroughly to eliminate the residual odor. Testing was performed in a temperature, noise and light controlled room.

2.4.3 Light and Dark Model (LD):
The method developed by Crawley and Goodwin (1980) [23], based on the natural aversion of animal for brightly lit places was adopted. Briefly, in a two compartment box, one dark and one brightly lit, the time spent in the light compartment and the crossings between the light and dark compartment was recorded for a test time of 5 min. After each test, the apparatus was sprayed with dilute alcohol and wiped thoroughly to eliminate the residual odor. Testing was performed in a temperature, noise and light controlled room.

2.4.4 Hole Board Test (HBT):
The apparatus was composed of a gray wooden box (50 cm×50 cm× 50 cm) with four equidistant holes 3 cm in diameter in the floor. The centre of each hole was 10 cm from the nearest wall of the box. The floor of the box was positioned 15 cm above the ground and divided into squares of 10 cm×10 cm with a water resistant marker. An

animal was placed in the center of the hole-board and allowed to freely explore the apparatus for 5 min. The numbers of squares crossed, the number of head-dipping and fecal contents were recorded. A head dip was scored if both eyes disappeared into the hole [24].

2.5 Statistical Analysis:
One specific group of animal was assigned to one specific drug treatment condition and each group comprised of six (n=6) animals. All the values are expressed as mean ± S.E.M. The data were analyzed by one-way ANOVA followed by Dunnett's test using GraphPad InStat 3. $P<0.05$ was considered as statistically significant.

3. Results
3.1 Effect of 4a on EPM:
The anxiolytic activity of 4a was evaluated using EPM model. The exploratory parameters; number of entries and time spent in open arms were significantly increased by 4a as compared to the vehicle control group $P<0.05$. Further, the effect of the 4a was dose dependent (2 and 4 mg/kg i.p.) (Table 1).

Table 1: Effect of 4a on EPM

Groups	No. of entries in open arm	Time in open arm (s)
Vehicle control	1.50 ± 0.56	67.33 ± 6.75
4a (2 mg/kg i.p.)	6.17 ± 1.08*	106.33 ± 7.08*
4a (4 mg/kg i.p.)	7.83 ± 1.66**	134.67 ± 9.76**
Diazepam (2 mg/kg i.p.)	15.17 ± 1.38**	171.67 ± 10.13**

All the values are expressed as mean ± S.E.M., *$P<0.05$, **$P<0.01$ as compared with vehicle control group to 4a treated animals, n = 6/group.

3.2 Effect of 4a on OFT:
4a dose dependently (2 and 4 mg/kg i.p.) increased the number of central and peripheral ambulation along with the time spent in central

area and number of rearings, significantly as compared to vehicle control group. $P<0.05$ was consider as statistically significant (Table 2).

Table 2: Effect of 4a on OFT

Groups	No. of peripheral ambulation	No. of central ambulation	Time in central area (s)	No. of rearings
Vehicle control	88.67 ± 7.54	3.83 ± 0.54	6.33 ± 1.20	3.67 ± 1.02
4a (2 mg/kg i.p.)	135.67 ± 8.91*	11.50 ± 2.23*	17.00 ± 2.00 *	11.00 ± 1.44*
4a (4 mg/kg i.p.)	166.00 ± 18.54**	19.50 ± 2.43**	25.50 ± 4.69**	13.67 ± 2.11**
Diazepam (2 mg/kg i.p.)	187.33 ± 8.53**	29.17 ± 2.06**	28.33 ± 2.58**	26.17 ± 1.64**

All the values are expressed as mean ± S.E.M., *$P<0.05$, **$P<0.01$ as compared with vehicle control group to 4a treated animals, n = 6/group

3.3 Effect of 4a on LD model:

4a dose dependently (2 and 4 mg/kg i.p.) reduced the latency to enter dark chamber whereas, increased the number of transitions and time in light chamber significantly compared to vehicle control group. $P<0.05$ was consider as statistically significant (Table 3).

Table 3: Effect of 4a on LD model

Groups	Latency to enter dark chamber (s)	Time in light chamber (s)	No. of transitions
Vehicle control	29.50 ± 3.89	59.00 ± 11.11	6.83 ± 1.33
4a (2 mg/kg i.p.)	19.17 ± 1.97*	113.83 ± 10.43**	12.50 ± 1.67*
4a (4 mg/kg i.p.)	14.83 ± 1.82**	136.00 ± 11.42**	14.17 ± 1.14**
Diazepam (2 mg/kg i.p.)	6.17 ± 1.17**	175.67 ± 4.36**	21.17 ± 1.42**

All the values are expressed as mean ± S.E.M., *P<0.05, **P<0.01 as compared with vehicle control group to 4a treated animals, n = 6/group.

3.4 Effect of 4a on HBT:

4a dose dependently (2 and 4 mg/kg i.p.) increased the number of crossing and the head dips, significantly as compared to vehicle control group. $P<0.05$ was consider as statistically significant (Table 4).

Table 4: Effect of 4a on HBT

Groups	No. of head dips	No. of crossings
Vehicle control	9.67 ± 1.12	9.33 ± 0.76
4a (2 mg/kg i.p.)	16.67 ± 1.99*	19.50 ± 1.61*
4a (4 mg/kg i.p.)	23.83 ± 2.12**	27.00 ± 4.22**
Diazepam (2 mg/kg i.p.)	37.00 ± 1.90**	58.67 ± 3.04**

All the values are expressed as mean ± S.E.M., *P<0.05, **P<0.01 as compared with vehicle control group to 4a treated animals, n = 6/group.

4. Discussion

Anxiety is a psychological disorder, which alters the behavior of an individual. Taking into view, about the current status of anxiety, it is the time for researcher to look into it quiet seriously and to come up with novel therapeutics. Hence, with this perspective, the present study was designed to investigate the anxiolytic profile of a novel 5-HT$_3$ receptor antagonist 4a by using behavioral models of anxiety in mice like, EPM, OFT, LD and HBT.

EPM is one of the most etiologically valid models for screening anxiolytics as it introduces an animal to an entirely new environment, open spaces and fear of balancing on a narrow platform [25]. It is well established that a substance showing anxiolytic activity raises the time spent and entries in open arm. Accordingly in the present study, 4a increased the number of entries and the time spent in open arms, which is an indicator of anxiolytic potential of 4a.

OFT is another reliable model for anxiety, where an animal is again introduced to a new and wide open space which leads to anxiety like feeling in animals triggered by two factors: individual testing and agoraphobia [26]. Anxiety is confirmed in animals as it moves to the periphery more. With this aspect, 4a increased the crossing centrally as well as peripherally, along with the time in central area of apparatus proving its anxiolytic effect.

LD model is a model for anxiety which is highly practiced at laboratory levels. In this model anxiety like behavior in animal is seen because of the novel environment and light which leads spontaneous exploratory behavior in animals [27]. It is reported that an animal spending more time in light chamber along with increased transition number is considered to show anxiolytic activity

[27]. In the present study, 4a increased the latency to enter the dark chamber, time spent in light chamber and number of transitions (that refers to the entry of the animal from one chamber to another). The increased latency to enter in the dark chamber when initially kept in brightly illuminated light chamber indicates the anxiolytic effect of the compound as the onset of anxiety of the animal is decreased. Further, the increased time spent in light chamber indicated less anxiety behavior and increased transitions showed increased exploratory behavior of the animal treated with 4a, suggesting anti-anxiety activity of 4a.

HBT is a model for anxiety, where an animal is introduced to an unfamiliar environment and is used mostly to asses anxiety, emotionality and response to stress [28]. The head-dipping behavior reflects the sensitivity towards the changes in emotional state of the animal and provides information that a fearless state in animals may be reflected by the raised head dipping behavior [29]. Likewise, 4a successfully raised the head dipping behavior and the score if visiting the squares on the hole board apparatus indicating the anxiolytic activity of 4a.

The mechanism perspective of 5-HT_3 antagonist for anxiolytic effect is still not cleared. Well, the information on role of serotonin in the anxiety and related disorder is now cleared. Some of the reports earlier suggests, that 5-HT_3 antagonist by blocking the post-synaptic receptors, makes serotonin available to act on other 5-HT receptors like 5-HT_1 and 5-HT_2 [30,31].

Conclusively, the present study aimed at investigating the anxiolytic potential of 4a in animal models, namely EPM, OFT, LD and HBT with standardized behavioral parameters. Based on the results obtained, we say that, exposure to the pre-clinical model produced anxiety behavior in animals which was significantly attenuated by 4a. Hence, 4a can be a potential drug candidate for anxiety disorder. Our further finding will deal with the molecular aspect involved with anxiolytic potential of 4a.

6. References
1. Sternbach LH, Reeder E. Quinazolines and 1,4-benzodiazepines. The rearrangement of chloro-2-chloromethyl-4-phenylquinazolines 3-oxide into 2-amino derivatives of 7-chloro-5-phenyl-3H-1,4-benzodiazepines 4-oxide. J Org Chem 1961; 26: 1111-1118.
2. Randall LO, Heise GA, Schallek W, Bagdon RE, Banziger RF, Boris A, Moe RA, Abrams WB. Pharmacological and clinical studies on VALIUM, a new psychotherapeutic agent of the benzodiazepine class. Curr Ther Res 1961; 3: 405-425.
3. Rall TW. Hypnotics and sedatives ethanol. In The Pharmacological Basis of Therapeutics (eds Gilman AG, Rall TW, Nies AS, Talyor P.) pp. 345-382. New York, Pergamon Press.
4. Griebel G. 5-Hydroxytryptamine-interacting drugs in animal models of anxiety disorders: more than 30 years of research. Pharmacol Ther 1995; 65(3): 319-395.
5. Millan, M.J. The neurobiology and control of anxious states. Prog Neurobiol 2003; 70(2): 83-244.
6. Soderpalm B, Engel JA. Serotoninergic involvement in conflict behaviour. Eur Neuropsychopharmacol 1990; 1:7-13.
7. Barnes NM, Costall B, Ironside JW, Naylor RJ. Identification of recognition sites in human brain tissue using [³H] zacopride. J Pharm Pharmacol 1988; 40: 668.
8. Kilpatrick GJ, Jones BJ, Tyers MB. Identification and distribution of 5-HT3 receptors in rat brain using radioligand binding. Nature 1987; 330: 24-31.
9. Costall B, Domeney AM, Gerrard PA, Kelly ME, Naylor RJ, Tyers MB. Effects of 5-HT receptor antagonists GR38032F, ICS205-930 and BRL43694 in test for anxiolytic activity. Br J Pharmacol 1988b; 93: 195.
10. Jones BJ, Costall B, Domeney AM, Kelly ME, Naylor RJ, Oakley NR, Tyers MB. The potential anxiolytic activity of GR38032F, a 5-HT_3

receptor antagonist. Br J Pharmacol 1988; 93: 985-993.

11. Hewlett W, Schmid SP, Salomon RM. Pilot trial of ondansetron in the treatment of eight patients with OCD. Presented at American Psychiatric Association Annual Meeting, Washington, DC, Abstr. NR682, 1999a.

12. Greist JH, Jefferson JW. Pharmacotherapy for obsessive– compulsive disorder. Br J Psychiatry 1998; 173(Suppl. 35): 64-70.

13. Hewlett WA. Benzodiazepines in the treatment of obsessive compulsive disorder. In: Goodman WA, Rudorfer MP, Maser JD, editors. Obsessive compulsive disorder, contemporary issues in treatment. New York: Lawrence Erlbaum Associates, 2000. pp. 405-429.

14. Lecrubier Y, Puech AJ, Azcona A, Bailey PE, Lataste X. A randomized double-blind placebo-controlled study of tropisetron in the treatment of outpatients with generalized anxiety disorder. Psychopharmacology 1993; 112: 129-133.

15. Schneier FR, Garfinkel R, Kennedy B, Campeas R, Fallon B, Marshall R, O'Donnell L, Hogan T, Liebowitz MR. Ondansetron in the treatment of panic disorder. Anxiety 1996; 2: 199-202.

16. Depot M, Caille G, Mukherjee J, Katzman MA, Cadieux A, Bradwejn J. Acute and chronic role of 5-HT3 neuronal system on behavioral and neuroendocrine changes induced by intravenous cholecystokinin tetrapeptide administration in humans. Neuropsychopharmacology 1999; 20: 177-187.

17. McCann UD, Morgan CM, Geraci M, Slate SO, Murphy DL, Post RM. Effects of the $5-HT_3$ antagonist, ondansetron, on the behavioral and physiological effects of pentagastrin in patients with panic disorder and social phobia. Neuropsychopharmacology 1997; 17: 360-369.

18. Zhang Z, Schmidt DE, Paulis T, Trivedi BL, Onaivi ES, Ebert MH, Hewlett WA. Anxiolytic-like effects of DAIZAC, a selective high-affinity $5-HT_3$ receptor antagonist, in the mouse elevated plus-maze. Pharmacol Biochem Behav 2001; 69: 571-578.

19. Mahesh R, Kumar K, Jindal A, Bhatt S, Devadoss T, Pandey DK. Antidepressant-like activity of (4-phenylpiperazin-1-yl) (quinoxalin-2-yl) methanone (4a), a novel $5-HT_3$ receptor antagonist: An investigation in behavior-based rodent models of depression. Indian J Pharmacol 2012; 44: 560-565.

20. Mahesh R, Devadoss T, Pandey DK, Yadav SK. Quinoxalin-2-carboxamides: Synthesis and pharmacological evaluation as serotonin type-3 ($5-HT_3$) receptor antagonists. J Enzym Inhib Med Chem 2011; 26: 610-615.

21. Rodgers RJ, Cole JC, Aboualfa K, Stephenson LH. Ethopharmacological analysis of the effects of putative 'anxiogenic' agents in the mouse elevated plus-maze. Pharmacol Biochem Behav 1995; 52: 805-813.

22. Redmond AM, Kelly JP, Leonard BE. Behavioural and neurochemical effects of dizocilpine in the olfactory bulbectomized rat model of depression. Pharmacol Biochem Behav 1997; 58: 355-359.

23. Crawley JN, Goodwin FK. 1980. Preliminary report of a simple animal behaviour model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav 1980; 13: 167-170.

24. Wei XY, Yang JY, Wang JH, Wu CF. Anxiolytic effect of saponins from *Panex quinquefolium* in mice. J Ethnopharmacol 2007; 111: 613-618.

25. Dawson GR, Tricklebank MD. Use of the elevated plus-maze in the search for novel anxiolytic agents. Trends Pharmacol Sci 1995; 16: 33-3636.

26. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003; 463: 3-33.

27. Imaizumi M, Suzuki T, Machida H, Onodera K. A fully automated apparatus for a light/dark test measuring anxiolytic or anxiogenic effects of drugs in mice. Jpn J Psychopharmacol 1994; 14: 83-91.

28. Nolan NA, Parkes MW. The effects of benzodiazepines on the behavior of mice on a hole board. Psychopharmacologia 1973; 29: 277-286.

29. Takeda H, Tsuji M, Matsumiya T. Changes in head-dipping behavior in the hole-board test reflect the anxiogenic and/or anxiolytic state in mice. Eur J Pharmacol 1998; 350: 21-29.

30. Greenshaw AJ. Behavioral pharmacology of 5-HT, receptor antagonists: A critical uptake on

therapeutic potential. Trends Pharmacol Sci 1992; 14: 265-270.

31. Kilpatrick GJ, Butler A, Ireland SJ, Michel AD, Tyers MB. Affinities of 5-HT uptake inhibitors for 5-HT, receptors in both binding and functional studies. Br J Pharmacol 1989; 98: Suppl 859P.