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Original Article

Anxiolytic-like activity of serotonin-3 receptor antagonist: In-silico molecular modeling, drug-likeness and evaluation of anxiolytic activity

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Abstract

Serotonin-3 receptor antagonists have a significant impact in treating nausea and vomiting, and various diseases of the central nervous system. Patients receiving chemotherapy have anxiety as one of their complications. Therefore, we explored the anxiolytic effect of the test compound, 6d {2-[4-(prop-2-en-1-yl)piperazin-1-yl]-1,8-naphthyridine-3-carbonitrile}, and the standard 5-HT_3 receptor antagonist, ondansetron. In this research, we carried out computational study to predict drug-likeness and bioactivity using online prediction tools. In-silico molecular docking studies were performed with the four conformations of 5-HT_3 receptors using AutoDoc Vina software and binding interactions were analyzed using Biovia Discovery Studio. Anxiolytic studies were conducted in mice using various animal models. Both the test and standard drug satisfied Lipinski and Veber rules, showed high oral absorption and blood-brain barrier permeability. In the toxicity prediction, the test compound showed higher LD_{50} compared to ondansetron and did not display other toxicities, whereas ondansetron exhibited mutagenicity. Docking studies revealed that the test compound has higher binding affinity with F, I1 and T conformations and lesser binding affinity with I2 conformation compared to ondansetron. Anxiolytic evaluation disclosed that the test compound and ondansetron exhibited significant anxiolytic activity at 10 mg/kg dose compared to saline control.

Keywords: 5-HT_3 receptor antagonist, anxiolytics, molecular docking, ADMET

1. Introduction

Serotonin (5-HT, 5-hydroxytryptamine) is one of the monoamine neurotransmitters that regulate numerous activities of the central nervous system (CNS) (Dutton & Barnes, 2008; Fakhfouri, Rahimian, Dyhrfjeld-Johnsen, Zirak & Beaulieu, 2019). The 5-HT_3 receptor acts through a pentameric ligand-gated ion channel, although all 5-HT receptors are G-protein coupled. Several preclinical and clinical research have shown that 5-HT_3 receptor antagonists (5-HT_3 RAs) like granisetron, tropisetron and ondansetron can successfully treat nausea and vomiting induced by radiotherapy, chemotherapy and postoperative conditions, and may alleviate the symptoms of irritable bowel syndrome (Thompson & Lummis, 2007; Walstab, Rappold & Niesler, 2010). Some studies reported that there is a prevalence of anxiety among cancer patients (Grassi *et al*., 2013; Nikbakhsh, Moudi, Abbasian & Khafri, 2014). It will be beneficial for the patients if the 5-HT_3 RAs reduce the nausea and vomiting, and also have the anxiolytic effect. Moreover, numerous studies reported that various CNS disorders viz. anxiety, depression, etc. are regulated by 5-HT_3 receptors (Fakhfouri *et al*., 2019; Machu, 2011) and also recent studies revealed the anxiolytic effect of ondansetron along with other 5-HT_3 RAs (Amir *et al*., 2020; Juza *et al*., 2020). So, in this study we explored the anxiolytic potential of our previously reported 5-HT_3 RA, 2-[4-(prop-2-en-1-yl)piperazin-1-yl]-1,8-naphthyridine-3-carbonitrile (6d) (Figure1) (Mahesh, Perumal

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& Pandi, 2004). In the homology model, acetylcholine-binding protein (AChBP), was used to study the binding affinity of 5-HT_3 RAs (Kesters, 2013). Recently the crystal structure of four conformations of 5-HT_3 receptor was reported (Polovinkin, 2018). In the present study, we performed computer-based molecular modeling to determine the binding affinity of the 5-HT_3 RA (6d), and ondansetron with the various conformations of 5-HT_3 receptor, predicted the pharmacokinetic profile, bioactivity and toxicity using online tools, and screened for its anxiolytic effect using various animal models.

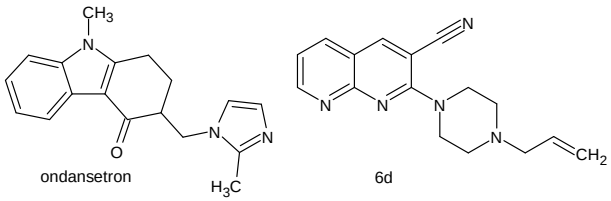


Figure 1. Structures of standard 5-HT3 RA, ondansetron and test compound, 6d

2. Materials and Methods

2.1 In-silico prediction

Computational research was performed on the test drug, 6d, and the standard 5-HT_3 RA, ondansetron, to predict its ADME properties, viz. Lipinski's rule (Lipinski, Lombardo, Dominy & Feeney, 1997), Veber rule (Veber *et al*., 2002) and topological polar surface area (TPSA) using SwissADME online tool (Daina, Michielin & Zoete, 2017). Percentage absorption was computed from TPSA using the published method (Zhao *et al*., 2002). The test drug, 6d, and standard drug, ondansetron, was subjected to bioactivity prediction using Molinspiration online tool (Molinspiration) and their results are shown in Table 2. To forecast the toxicity of the test and standard drugs, the online tool ProTox-ii was used (Drwal, Banerjee, Dunkel, Wettig & Preissner, 2014).

2.2 In-silico molecular docking

The 2D structures (.mol) of test compound, 6d, and the standard 5-HT_3 RA, ondansetron, were drawn (Figure 1), converted to 3D structure (.pdb) and each molecule was energy minimized using Chem Office tool. The docking analysis was then run with the energy-minimized ligands as input. The four conformations of protein target, mouse 5-HT_3 receptor, serotonin-bound, F conformation (PDB id: 6HIN), mouse 5-HT_3 receptor, serotonin-bound, I1 conformation (PDB id: 6HIO), mouse 5-HT_3 receptor, serotonin-bound, I2 conformation (PDB id: 6HIQ) and mouse 5-HT_3 receptor, tropisetron-bound, T conformation (PDB id: 6HIS), were taken from the Protein Data Bank. Protein preparation was done in Biovia Discovery Studio 2020. Water molecules were deleted from the protein complex; bound ligand was selected to find the binding site attributes, and the ligand molecule was deleted from the complex. Polar hydrogen atoms and required charges were added to the protein. Redocking of bound ligand with the target protein was carried out to validate the binding sites. The required format (.pdbqt) of the protein and the ligand for docking was created using Auto Doc Vina (MGLTools-1.5.6), and docking was carried out (Trott & Olson, 2010). During the docking process, twenty conformers were created for each ligand along with their binding energy. The most favorable conformation, with least binding energy, was selected to find the interactions between the receptor and ligand using Biovia Discovery Studio 2020.

2.3 Anxiolytic screening

The Institutional Animal Ethics Committee granted approval to the animal studies. Swiss albino mice (20-25 gm) were housed under normal laboratory conditions in a controlled environment (temperature 25º ± 2 ºC; humidity 60% ± 10%), maintaining a 12-hr light-dark cycle, free access to water and food as per CPCSEA guidelines. The day before the experiment, the animals were kept in groups of six in plexiglass cages in the laboratory. The experiment was conducted between 8 a.m. and 12 noon, during the light phase of the cycle. Diazepam (0.2 mg/kg) was used as a positive control; saline as a negative control; ondansetron and test compound 6d were used at doses of 0.1, 1.0 and 10 mg/kg. The drug was dissolved in saline and injected into the intraperitoneal cavity (i.p.) 1 hr. before the test. The experimental data were statistically analysed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad InStat 3 software. Statistical significance was established at $p < 0.05$. The results of anti-anxiety studies are expressed as mean ± SEM.

2.3.1 Elevated plus maze

Two oppositely open (30 x 5 cm) and two oppositely closed arms of the same size make up the EPM. The latter is enclosed on all sides by ten-centimeter walls, but it is open; the open arms have no lips. In the middle section, the four arms are separated (5 x 5 cm). The entire apparatus is elevated from the ground by 30 centimeters. During the 5-minute test, the mouse was placed alone in the maze's center and its behaviour was watched. Time spent in the maze's open arms (expressed as a percentage of total time spent in the open arms), and entries into the open arms (expressed as a percentage of total entries in the open arms); time spent in the device's center is ignored (Hogg, 1996; Rodgers & Cole, 1994). The results were compared to the positive control and the vehicle control groups.

2.3.2 Light-dark box

The device is a rectangular box with an open top (46 x 27 x 30 cm high) made of plexiglass. It is divided into a large compartment (27 x 27 cm) and a small compartment (18 x 27 cm), with a 7.5 x 7.5 cm opening in the center of the floor partition. The large compartment is painted white and has a 60-watt light source, whilst the small compartment is black and has a faint red (60-watt) lamp. The mouse was placed alone in the light chamber and the following parameters recorded within 5 minutes; time spent in each compartment and shuttle between the two compartments. A mouse with all four legs in any compartment was considered to have an exchange compartment. After each test, the basewas washed with 20% v/v ethanol and dried (Bourin &

Hascoët, 2003; Hascoët & Bourin, 1998). The results obtained were compared with the vehicle and positive control groups.

2.3.3 Hole board test

It consists of a plexiglass (blackened) square plate (50 x 50 cm) with a diameter of 3 cm and 16 equidistant holes. With the help of a wooden frame, the plank is 20 cm above the ground. The board is divided into 25 squares to measure locomotor activity. The mouse was then placed alone in the device's center and given five minutes to explore it. The exploration score is determined by the number of head dips in the hole, and the number of squares passed by the hind legs is used as the animal's locomotion score (File & Wardill, 1975). The results obtained were compared with the vehicle and positive control groups.

3. Results and Discussion

3.1 Computational study

From all the calculated parameters using SwissADME (Daina *et al*., 2017), it has been observed that both standard 5-HT_3 RA, ondansetron, and the test compound, 6d, satisfy Lipinski (Lipinski *et al*., 1997) and Veber (Veber *et al*., 2002) rules with zero violations (Table 1). Ondansetron (% ABS, 95.26%) and 6d (% ABS, 89.66%) showed high oral absorption, and BBB permeability, which make the compounds acting on the CNS as an anxiolytic. Molinspiration bioactivity prediction (Molinspiration) showed ondansetron and 6d have equal affinity with GPCR ligand, whereas 6d has lesser affinity for nuclear receptor ligand, enzyme inhibitor, ion channel modulator and protease inhibitor, and higher affinity for kinase inhibitor compared to ondansetron (Table 2). Toxicity prediction by ProTox-ii (Drwal *et al*., 2014) showed higher LD_{50} for 6d (300 mg/kg) than ondansetron (95 mg/kg). Moreover, 6d exhibited no toxicity, whereas ondansetron exhibited mutagenicity (Table 3). Despite the fact that ondansetron has not been reported for its mutagenicity in preclinical or clinical investigations (Villikka, Kivistö, & Neuvonen, 1999), this prediction showed mutagenicity, but the other predictions were accurate.

3.2 In-silico molecular docking

We performed docking study with the four conformations (F, I1, I2, and T) of 5-HT_3 receptor. The test compound, 6d, showed higher binding affinity than ondansetron with F (-7.9 vs. -7.0 kcal/mol), I1 (-7.8 vs. -6.9 kcal/mol) and T (-8.4 vs. -7.7 kcal/mol) conformations and lesser affinity with I2 (-6.7 vs. -7.0 kcal/mol) conformation (Table 4). The test compound, 6d, interacts with F, I1 and T conformations of 5-HT_3 receptors with least energy compared to ondansetron. 6d and ondansetron showed hydrogen bonding and hydrophobic interactions with the amino acids of F, I1, I2 and T conformations of 5-HT_3 receptor as shown in Table 4. Both 6d and ondansetron showed hydrophobic interactions with amino acids; Trp-63 and Tyr-126 of F conformation. Both compounds showed hydrogen bonding with Tyr-64 and hydrophobic interaction with Trp-63 of I1. In I2 conformation the compounds showed hydrophobic interaction with Trp-156 and Phe-199, whereas in T conformation the compounds showed hydrophobic interactions with Trp-63, Ile-44 and Arg-65. The 3D and 2D binding interactions of the test compound, 6d, and the standard, ondansetron, with the 5-HT_3 receptor are depicted in Figures 2 and 3.

Table 1. ADME properties prediction using Swiss ADME

S.No.	Properties	6d	Ondansetron
1	M.Wt	279.34	293.36
2	NHD	0	0
3	NHA	4	2
4	cLogP	1.88	2.54
5	Lipinski rule violation	0	0
6	RBN	3	2
7	TPSA ($Å^2$)	56.05	39.82
8	Veber rule violation	0	0
9	% ABS	89.66	95.26

M.Wt, molecular weight; NHD, no. of hydrogen donor; NHA, no. of hydrogen acceptor; RBN, no. of rotatable bonds; TPSA, topological polar surface area; ABS, absorption

Table 2. Bioactivity prediction using Molinspiration

S.No.	Bioactivity	6d	Ondansetron
1	GPCR ligand	0.27	0.27
2	Ion channel modulator	0.12	0.22
3	Kinase inhibitor	0.35	-0.05
4	Nuclear receptor ligand	-0.42	-0.38
5	Protease inhibitor	-0.46	-0.31
6	Enzyme inhibitor	0.21	0.29

3.3 Anxiolytic Screening

3.3.1 Elevated plus maze (EPM)

The EPM is a popular anxiety animal model in which rodents are inherently afraid of open space and height. Spatio-temporal are the primary indices of anxiety in the EPM. The number of open arm entries is expressed as % open arm entries, and the time spent in the open arm is expressed as % time in open arm (Rodgers & Cole, 1994; Hogg, 1996). The response of drugs on % entries in open arms is shown in

Table 3. Toxicity prediction, computed by ProTox-ii

	LD_{50} (mg/kg)	Toxicity class	Toxicity				
			Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
Ondansetron	95	3	No	No	No	Yes	No
6d	300	3	No	No	No	No	No

Table 4. Binding affinity and amino acid interactions of 6d and ondansetron

S.No.	5-HT$_3$ receptor conformation	Ligand	Binding affinity (kcal/mol)	Interactions	
				Hydrogen bonding	Hydrophobic bonding
	F	6d	-7.9	Tyr-114 (C-H)	Ile-112 (π-σ, π-Alkyl), Pro-128; Arg-65 (Alkyl), Trp-63; Tyr-126 (π-Alkyl)
		Ondansetron	-7.0	-	Trp-63; Tyr-126 (π-π staked)
	I1	6d	-7.8	Tyr-64 (C-H)	Pro-128; Ile-44; Arg-65 (Alkyl), Trp-63; Ile-112 (π-Alkyl)
		Ondansetron	-6.9	Tyr-64	Trp-63 (π-σ), Tyr-126 (π-π stacked), Ile-44 (π-Alkyl)
	I2	6d	-6.7	Asn-101	Trp-156; Phe-199 (π-π stacked)
		Ondansetron	-7.0	Thr-152, Glu-102 (C-H), Phe-199 (π-donor H-bond)	Trp-156 (π-π stacked, π-Alkyl), Phe-199 (π-π stacked)
	T	6d	-8.4	-	Trp-63 (π-σ), Tyr-126 (π-π stacked), Ile-44 (Alkyl), Pro-128; Arg-65 (π-Alkyl)
		Ondansetron	-7.7	-	Ile-44 (π-σ, π-Alkyl, Alkyl), Trp-63 (π-σ), Arg-65 (Alkyl, π-Alkyl)

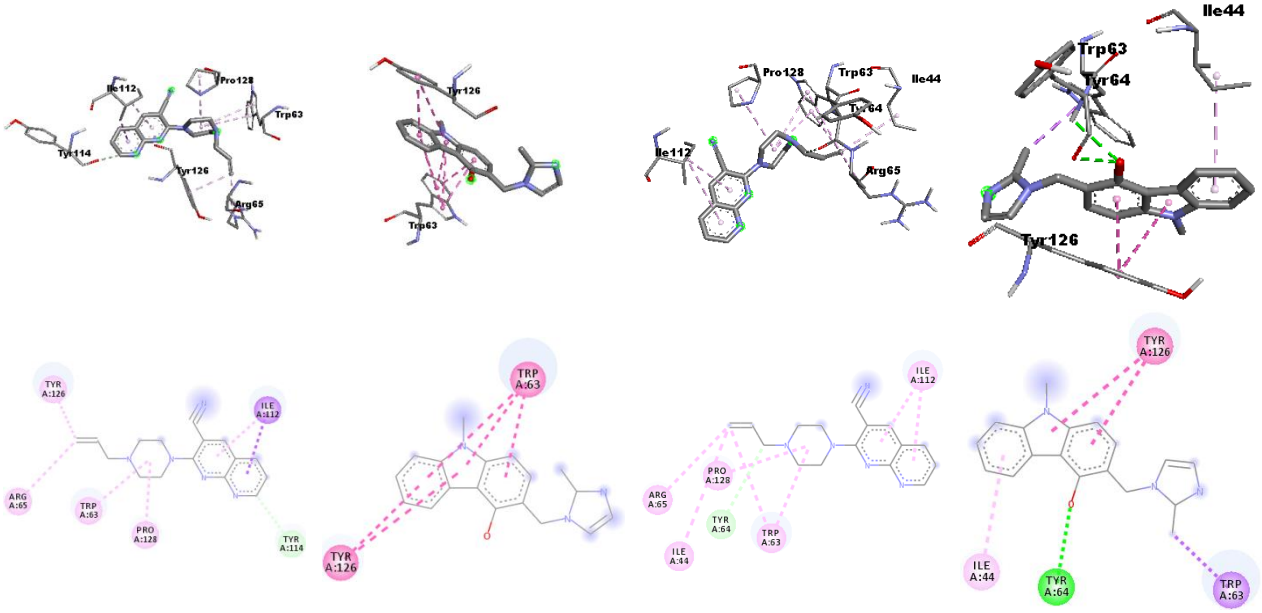
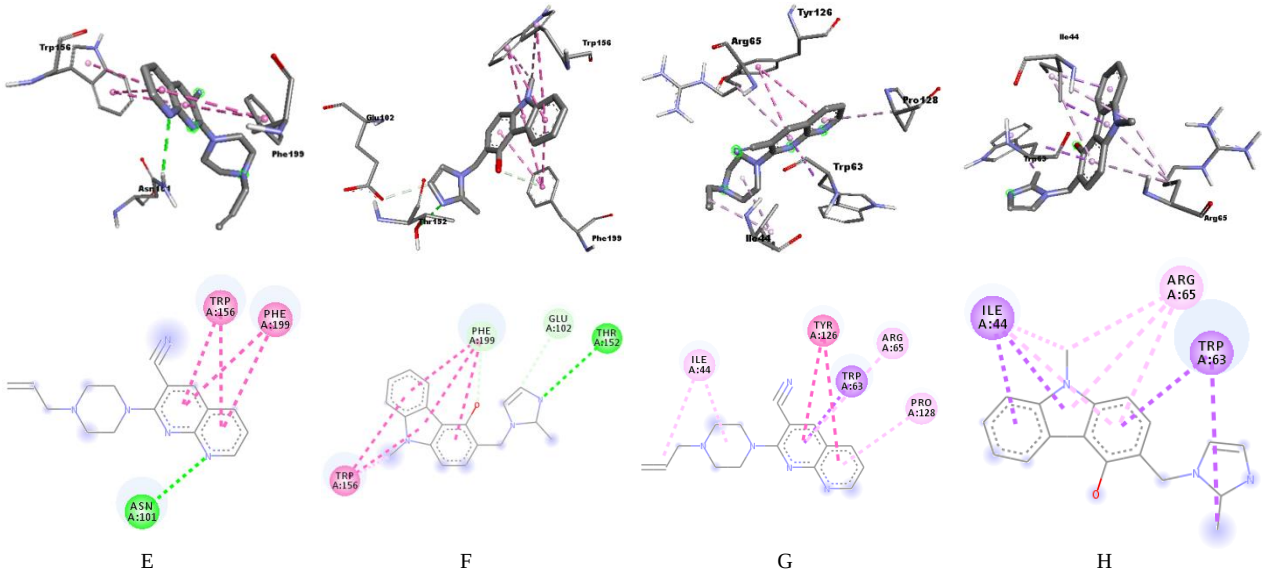


Figure 2. Binding interaction (3D and 2D) with F and I1 conformation of 5-HT3 receptor: A, 6d with F conformation; B, ondansetron with F conformation; C, 6d with I1 conformation; D, ondansetron with I1 conformation. Green line represents hydrogen bonding interactions and pink line represents hydrophobic interactions.

Table 5. ANOVA showed extremely significant difference among the given treatments [F (7, 40) = 7.01, p < 0.0001]. Tukey's test revealed that diazepam (0.2 mg/kg), ondansetron (10 mg/kg) and 6d (10 mg/kg) significantly increased % entries in open arm, whereas the lower doses (1.0 & 0.1 mg/kg) of ondansetron and 6d did not alter the measure significantly in comparison to saline control. The compound 6d did not show significant difference in % entries in open arm compared to ondansetron and diazepam at the various doses tested. The response of drugs on % time spent in open arms is given in Table 5. ANOVA showed extremely significant difference among the given treatments [F (7, 40) = 5.84, p = 0.0001]. Tukey's test revealed that diazepam (0.2 mg/kg), ondansetron (10 mg/kg) and 6d (10 mg/kg) significantly (p < 0.05) increased the % time spent in open arms, whereas at the lower doses (1.0 & 0.1mg/kg) neither ondansetron nor 6d altered the measure significantly in comparison to saline control. These findings are consistent with the previously published approach (Hogg, 1996).

3.3.2 Light-dark box

This model is primarily based on the concept that rodents have a natural aversion to brightly lit environments. The amount of time the animal spends in the bright compartment and the frequency of crossings among the bright and dark compartments reveal the animal's light/dark preference. Since anti-anxiety drugs should diminish the natural aversion to light, the basic characteristic of this model is that anti-anxiety drugs increase the frequency of crossings and/or the time spent in the light compartment. The last parameter is typically considered to be the most relevant



E F G H

Figure 3. Binding interaction (3D and 2D) with I2 and T conformation of 5-HT3 receptor: E, 6d with I2 conformation; F, ondansetron with I2 conformation; G, 6d with T conformation; H, ondansetron with T conformation. Green line represents hydrogen bonding interactions and pink line represents hydrophobic interactions.

Table 5. Effect of drugs on % open arm entries and % time spent in open arm (EPM)

Treatment	% Open arm entries	% Time in open arm
Control	6.11 ± 3.89	0.45 ± 0.29
Diazepam (0.2 mg/kg)	27.5 ± 5.09*	2.34 ± 0.45*
Ondansetron (0.1 mg/kg)	6.95 ± 4.52	0.55 ± 0.35
Ondansetron (1 mg/kg)	7.94 ± 5.59	0.56 ± 0.37
Ondansetron (10 mg/kg)	30.14 ± 3.9*	2.33 ± 0.42*
6d (0.1 mg/kg)	6.55 ± 4.37	0.49 ± 0.31
6d (1 mg/kg)	8.33 ± 5.7	0.63 ± 0.4
6d (10 mg/kg)	34 ± 3.42**	2.21 ± 0.4*

Values show mean ± SEM; n = 6. *indicates $p < 0.05$;
**indicates $p < 0.01$ when compared to vehicle control group

Table 6. Effect of drugs on no. of transitions and % time spent in lit area (light-dark box)

Treatment	No. of transitions	% time in lit area
Control	8.83 ± 0.48	20.17 ± 2.06
Diazepam (0.2 mg/kg)	12 ± 0.68*	32.5 ± 3.02*
Ondansetron (0.1 mg/kg)	9.33 ± 0.71	24 ± 2.82
Ondansetron (1 mg/kg)	10.5 ± 0.67	26.5 ± 2.46
Ondansetron (10 mg/kg)	11.83 ± 0.54*	33 ± 2.79*
6d (0.1 mg/kg)	10 ± 0.86	26 ± 2.93
6d (1 mg/kg)	11.17 ± 0.6	29.5 ± 2.45
6d (10 mg/kg)	12 ± 0.58*	35 ± 2.41**

Values show mean ± SEM; n = 6. *indicates $p < 0.05$;
**indicates $p < 0.01$ when compared to vehicle control group

(Bourin & Hascoët, 2003; Hascoët & Bourin, 1998). The response of drugs on number of transitions among the two compartments is shown in Table 6. ANOVA showed very significant difference among the given treatments [$F(7, 40) = 3.64$, $p = 0.0039$]. Tukey's test revealed that diazepam (0.2 mg/kg), ondansetron (10 mg/kg) and 6d (10 mg/kg) significantly ($p < 0.05$) increased the number of transitions between the two compartments, whereas neither ondansetron nor 6d at lower doses (1.0 & 0.1 mg/kg) changed the measure significantly in comparison to saline control. The response of drugs on % time spent in light area is given in Table 6. ANOVA showed very significant variation among the given treatments [$F(7, 40) = 3.69$, $p = 0.0036$]. Tukey's test revealed that diazepam (0.2 mg/kg) ($p < 0.05$), ondansetron (10 mg/kg) ($p < 0.05$) and 6d (10 mg/kg) ($p < 0.01$) significantly increased the % time spent in light area, whereas the lower doses (1.0 & 0.1 mg/kg) of ondansetron and 6d did not alter the measure significantly at $p < 0.05$ in comparison to saline control. Similar finding was observed in a research study that ondansetron to have anxiolytic properties in a light-dark box (Costall *et al*., 1989).

3.3.3 Hole board test

Exploratory behaviour in rodents is measured in the hole board test (File & Wardill, 1975). The number of rodent head dips observed in the hole-board device has been accepted as a parameter for assessing the animal's anxiolytic status. In this model, it is reported that non-sedative doses of benzodiazepines and anti-anxiety drugs increase the frequency of head dips in mice. The effect of anti-anxiety drugs on locomotion can be assessed by the number of squares the animal passes through during the experiment (Crawley, 1985). Table 7 shows the effect of drugs on the number of head dips in the hole of the hole-board device. ANOVA showed very significant difference among the different treatments [$F(7, 40) = 4.33$, $p = 0.0012$]. Tukey's test revealed that diazepam (0.2 mg/kg) ($p < 0.05$), ondansetron (10 mg/kg) ($p < 0.05$) and 6d (10 mg/kg) ($p < 0.01$) significantly increased the number of head dips, whereas ondansetron and 6d at lower doses (1.0 & 0.1 mg/kg) did not change the measure significantly at $p < 0.05$ in comparison to saline control. The response of drugs on the frequency of line crossings is presented in Table 7.

Table 7. Effect of drugs on no. of head dips and no. of line crossings (hole board)

Treatment	No. of head dips	No. of line crossings
Control	21.17 ± 1.25	60 ± 7.51
Diazepam (0.2 mg/kg)	27.33 ± 1.17*	74.17 ± 6.33
Ondansetron (0.1 mg/kg)	21.83 ± 1.17	62.83 ± 6.22
Ondansetron (1 mg/kg)	24.17 ± 1.45	60.5 ± 7.93
Ondansetron (10 mg/kg)	27.33 ± 1.45*	65.33 ± 6.33
6d (0.1 mg/kg)	23.16 ± 1.30	61.83 ± 7.4
6d (1 mg/kg)	25.33 ± 1.48	63 ± 6.95
6d (10 mg/kg)	28.67 ± 1.28**	69 ± 7.58

Values show mean ± SEM; n = 6. *indicates $p < 0.05$;
**indicates $p < 0.01$ when compared to vehicle control group

ANOVA showed insignificant difference among the different treatments [$F(7, 40) = 0.4666$, $p = 0.853$]. This test clearly indicates that diazepam, ondansetron and 6d, at the given doses, did not affect the locomotor activity of the mice. In one study, ondansetron was found to exhibit anxiolytic characteristics in a battery of tests including the hole-board test (Gupta, Radhakrishnan, Thangaraj, & Kurhe 2015).

4. Conclusions

The test drug, 6d, and standard 5-HT_3 RA, ondansetron, showed drug-likeness property. The test drug exhibited better binding affinity with three conformations of 5-HT_3 receptors compared to ondansetron. The test and standard drugs did not show significant anxiolytic activity at lower doses (1.0 & 0.1 mg/kg) but at higher dose (10 mg/kg), both of them exhibited significant anxiolytic activity when compared to saline control. However, neither of them showed significant differences in anxiolytic activity compared to diazepam at the given doses. Further studies can be performed to establish the in-vivo binding affinity using radio-ligand binding studies.

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