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Original Article

Antidepressant-like effects of a novel 5-HT$_3$ receptor antagonist 6z in acute and chronic murine models of depression

Deepali GUPTA[1, *], Mahesh RADHAKRISHNAN[1], Yeshwant KURHE[1], Devadoss THANGARAJ[2], Visakh PRABHAKAR[1], Prateek KANADE[1]

[1]*Department of Pharmacy, Birla Institute of Technology & Science, Pilani, Rajasthan-333031, India;* [2]*KVSR Siddhartha College of Pharmaceutical Sciences, Vijaywada, Andhra Pradesh-520001, India*

Aim: To investigate the antidepressant-like effects of a novel 5-HT$_3$ receptor antagonist *N*-(benzo[d]thiazol-2-yl)-3-methoxyquinoxalin-2-carboxamide (6z) in acute and chronic murine models of depression.

Methods: 5-HT$_3$ receptor antagonism was examined in guinea pig ileum *in vitro*. A tail suspension test (TST) was used as acute depression model to evaluate the antidepressant-like behavior in mice treated with 6z (0.5–2 mg/kg, ip). In chronic depression model, mice were exposed to a 4-week chronic unpredictable stress (CUS) protocol, and treated with 6z (0.5–2 mg·kg^{-1}·d^{-1}, *po*) or a positive drug fluoxetine (10 mg·kg^{-1}·d^{-1}, *po*) in the last 2 weeks, followed by behavioral and biochemical assessments.

Results: The 5-HT$_3$ receptor antagonism of 6z (pA_2=7.4) in guinea pig ileum was more potent than that of a standard 5-HT$_3$ receptor antagonist ondansetron (pA_2=6.9). In acute depression model, 6z administration significantly decreased the immobility duration. In chronic depression model, 6z administration reversed CUS-induced depressive-like behavior, as evidenced by increased immobility duration in the forced swim test and sucrose preference in the sucrose preference test. Furthermore, chronic administration of 6z prevented CUS-induced brain oxidative stress, with significant reduction of pro-oxidant markers and elevation of antioxidant enzyme activity. Moreover, chronic administration of 6z attenuated CUS-induced hypothalamic-pituitary-adrenal axis hyperactivity, as shown by reduced plasma corticosterone levels. Similar results were observed in the fluoxetine-treated group.

Conclusion: 6z is a novel 5-HT$_3$ receptor antagonist with potential antidepressant-like activities, which may be related to modulating hypothalamic-pituitary-adrenal axis and attenuating brain oxidative damage.

Keywords: depression; antidepressant; 5-HT$_3$ receptor antagonist; carboxamide; fluoxetine; tail suspension test; chronic unpredictable stress; forced swim test; sucrose preference test; oxidative stress; hypothalamic-pituitary-adrenal axis

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Introduction

Depression is a widespread mental disorder that is often manifested by psychological, behavioral and physiological abnormalities. Depression is becoming one of the most prevalent public health problems because of its high rate of morbidity, recurrence and mortality, producing a serious burden at both the personal and social levels[1]. According to the World Health Organization, depression was ranked to be the third leading cause of global burden of disease in 2004 and will move into first place by 2030[2].

Despite a large increase in the number of antidepressants, the pharmacotherapy of depression remains inadequate[1]. At least 40% of patients do not respond to antidepressant therapy[3], although meaningful therapeutic effects are observed only after several weeks of treatment with existing antidepressants[4]. Additionally, most of the currently available agents are associated with frequent and persistent side effects, such as sedation, apathy and fatigue, sleep disturbances, cognitive impairments and sexual dysfunctions[5]. Therefore, there is a grave need for research and development of novel pharmacological agents with effective therapeutic efficacy and minimum side effects.

For decades, decreased central serotonergic tone has been associated with the pathogenesis of depression. Support

* To whom correspondence should be addressed.
E-mail deepaligupta2010@gmail.com
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of the serotonin (5-HT) hypothesis of depression is, in part, ascribed by the antidepressant action of selective serotonin reuptake inhibitors (SSRIs) and other monoamine-centered therapies[4]. 5-HT is a key neurotransmitter that regulates mood and emotional behavior by acting through its receptor and subsequently propagating the cascade of downstream events[6]. Fourteen 5-HT receptor subtypes belonging to seven major families have been identified in the brain, which have been reported to have behavioral effects[7]. For example, agonists at 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2C}, 5-HT_4, and 5-HT_6 receptors have been demonstrated to exhibit antidepressant-like effects[8–12]. Additionally, antagonists at 5-HT_{2A}, 5-HT_{2C}, 5-HT_6, and 5-HT_7 receptors have been reported to produce antidepressant-like responses similar to those of SSRIs[13–16]. Moreover, targeting specific 5-HT receptors may enhance the antidepressant response, decrease undesirable effects due to a non-specific increase in 5-HT activity and reduce the therapeutic lag associated with the current pharmacological agents[6].

In recent years, 5-HT_3 receptors have been identified as potential targets for antidepressant compounds[6]. 5-HT_3 receptors are widely expressed in discrete areas of the brain, which include brain stem nuclei and higher cortical areas, such as the amygdala, hippocampus and cortex, preferentially involved in the regulation of mood and behavioral activities[6]. The antagonism of 5-HT_3 receptors has shown significant antidepressant-like activity in several preclinical models. Ondansetron, a selective 5-HT_3 receptor antagonist, has shown antidepressant-like effects with a significant decrease in the duration of immobility during mouse forced swim test (FST) and tail suspension test (TST) and reversal of depressive behavior in olfactory bulbectomized rat model of depression[17]. Additionally, tropisetron and MDL 7222, the potential 5-HT_3 receptor antagonists, have shown antidepressant-like behavior in rodents exposed to FST and TST, respectively[18, 19]. Furthermore, the co-administration of 5-HT_3 antagonists has been reported to augment the antidepressant-like effects of SSRIs (such as fluoxetine), whereas currently existing antidepressants have been demonstrated to have antagonistic activity at 5-HT_3 receptors in the brain[20, 21].

Moreover, clinical studies have revealed that 5-HT_3 receptor antagonists can reverse depressive symptoms in humans[6]. Interestingly, 5-HT_3 receptor antagonists have demonstrated antidepressant-like effects in low-dose ranges and improvement in depression-related symptoms within 2–3 weeks of treatment[17]. Therefore, novel compounds targeting 5-HT_3 receptors may be more effective in treating depression-related disorders.

Several animal models of depression have been proposed to evaluate the antidepressant-like effects of novel compounds[22]. However, the selection of a model for an ideal antidepressant test battery is still a matter of debate[23]. Among acute models, TST is a robust testing paradigm that is regularly used in antidepressant screening protocols across laboratories[24]. It offers the advantages of being simple, straight-forward and sensitive to short-term antidepressant effects[23].

Chronic unpredictable stress (CUS) is a valid chronic model of depression that mimics many of the behavioral and biochemical consequences of human depression[25]. Chronic exposure to stress in mice has been reported to produce behavioral despair in FST (helplessness behavior) and anhedonia-like (inability to experience pleasure) behavior similar to that observed in depressed patients[26–28]. Biochemical studies have shown that CUS-induced oxidative brain damage is involved in the etiopathogenesis of depression[25]. Chronic stress results in increased production of reactive oxygen species (ROS), which are counteracted by an antioxidant defense mechanism. However, in situations where the generation of ROS exceeds the capacity of antioxidant defense, these excessive free radicals lead to neurocellular damage by enzyme inactivation, lipid peroxidation and DNA modifications[29, 30]. The oxidative stress profiles of depressed patients have demonstrated impairments in the antioxidant system, such as superoxide dismutase, catalase and glutathione peroxidase and higher products of lipid peroxidation than healthy controls[31], whereas several antidepressant agents have reported to reverse the increased oxidative load associated with depressive episodes[30].

The exposure to CUS induces alterations in hypothalamic-pituitary-adrenal (HPA)-axis functions, which are consistent with human depression[22]. It has been well reported that the pathophysiology of depression is linked to the hyperactivation of the HPA-axis[32], which is characterized by increased levels of circulating glucocorticoids resulting in hippocampal neurodegeneration and inducing depressive-like behavior in rodents, which in turn is effectively counteracted by treatment using antidepressants[33]. Therefore, it can be implied that increased brain oxidative stress and HPA-axis hyperactivity are considered to be involved in the pathogenesis of depression and that measuring these markers may provide a likely mechanism of action for the antidepressant effects of novel molecules.

Given all of these factors, a series of *N*-substituted-3-methoxyquinoxalin-2-carboxamides as 5-HT_3 receptor antagonists were designed using the ligand-based approach[34] and were synthesized from the starting material, *o*-phenylenediamine, in the sequence of reactions depicted in scheme 1 (supplementary data). The targeted new chemical entities were preliminarily screened for their antidepressant potential using FST. 6z, [N-(Benzo[d]thiazol-2-yl)-3-methoxyquinoxalin-2-carboxamide] (Figure 1) was selected because of its high antidepressant potential observed in preliminary testing. In the present study, a detailed investigation of the antidepressant-like effects of 6z was performed. First, the 5-HT_3 receptor

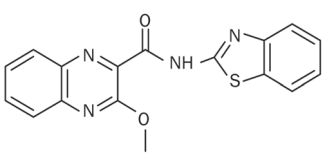

Figure 1. Structure of 6z [*N*-(Benzo[d]thiazol-2-yl)-3-methoxyquinoxalin-2-carboxamide].

antagonistic potential of the ligand was determined using longitudinal muscle myenteric plexus preparations from guinea pig ileum against a standard 5-HT_3 agonist, 2-methyl-5-HT, and its antagonism activity was expressed as pA_2 values[35], followed by an estimation of effective doses of 6z based on the dose response study[17]. Consequently, the evaluation of its antidepressant-like effects in validated acute (TST)[36] and chronic (CUS)[37] murine models of depression was performed. Furthermore, the likely mechanism of action of the ligand was determined by measuring the brain oxidative stress and plasma corticosterone (CORT) levels as a marker of dysregulated HPA-axis functions[30].

Materials and methods

Animals

Swiss albino mice (22–25 g, of either sex), male Dunkin Hartley guinea pigs (350–400 g) were obtained from Hisar Agricultural University, Haryana, India. The animals were housed in groups of six mice/cage (26 cm×19 cm×13 cm) and maintained in standard laboratory conditions with alternating light-dark cycle of 12 h each, temperature 23±2 °C and humidity conditions 62%±5% RH in the housing unit. The animals had free access to food (standard pellet chow feed) and filtered water *ad libitum*, except during the administration of the stress protocol. Behavioral testing was conducted during the light cycle; a separate group of the animals were used for all of the behavioral assays. The animals were treated according to the guidelines of the *Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, Registration number: 417/01/a/ CPCSEA)*, and all of the experiments were conducted in adherence to the approved protocol of the Institutional Animal Ethics Committee, Birla Institute of Technology & Science, Pilani, India (Protocol number: IAEC/RES/14/11/REV/06, August 2011).

Drugs

6z, [*N*-(Benzo[d]thiazol-2-yl)-3-methoxyquinoxalin-2-carboxamide] (Figure 1) was synthesized, and its structure was confirmed using infrared (IR) spectroscopy, mass spectrometry (MS) and proton nuclear magnetic resonance (H^1NMR) spectroscopy (Medicinal Chemistry Group, BITS-Pilani, Rajasthan, India). Fluoxetine (FLX), a SSRI, was obtained from Ranbaxy Research Laboratories, Haryana, India. 2-methyl-5-HT (a 5-HT_3 receptor agonist) was purchased from Tocris Biosciences, Bristol, UK. 6z (0.5–4 mg/kg) and FLX (10 mg/kg) were freshly prepared in distilled water and administered to the respected groups in a constant volume of 10 mL, whereas the control group received only the vehicle (distilled water) in the same volume.

5-HT_3 receptor antagonistic activity

For 5-HT_3 receptor antagonistic activity, guinea pigs were sacrificed by mild ether anesthesia followed by cervical dislocation. The abdomen was cut open, and a length of ileum, approximately 2 cm from the ileo-cecal junction, was excised. The longitudinal muscle-myenteric plexus (LMMP), measuring 3–4 cm in length, was removed and mounted according to a previously described method[35]. The tissue was equilibrated for 30 min under a resting tension of 500 mg and constant aeration in a 40 mL organ bath containing Tyrode's solution maintained at 37 °C. Non-cumulative concentrations (10^{-8}–10^{-4} mol/L) of 2-methyl-5-HT were added with a 15-min dosing cycle (to prevent desensitization) and left in contact with the tissue until the maximal contraction had developed. A fixed 2-methyl-5-HT concentration (10^{-5} mol/L), approximately ED_{50}, was used for the antagonism studies. To study the antagonist effect of the test compound on the response evoked by 2-methyl-5-HT, the compounds were added to the organ bath and left in contact with the tissue for at least 10 min before the addition of 2-methyl-5-HT. The contractions were recorded using a T-305 Force transducer coupled to Student's physiograph (Bio Devices, Ambala, India). Antagonism was quantitatively expressed in the form of pA_2 values, defined as negative logarithm of molar concentration of antagonist producing a 2-fold shift of the agonist concentration-activity curve[35].

Dose-response study

The dose-response profile of 6z was assessed using the mouse spontaneous locomotor activity (SLA). The SLA was assessed using actophotometer[17], which consisted of a dark square chamber (30 cm×30 cm) with inside walls painted black. The mice were individually placed in the chamber, and after an initial 2-min familiarization period, the digital locomotor scores were recorded for a subsequent 8-min period. The chamber was cleaned in dilute (70% *v*/*v*) alcohol and dried between trails. Two separate sets of experiments with 6z (0.5, 1, 2 and 4 mg/kg) were conducted to assess the reproducibility of the results. In the single-dose study, 30 min after 6z (0.5–4 mg/kg, ip), the mice were subjected to the locomotor activity test.

Behavioral assays

Acute study: tail suspension test

TST was conducted as described previously with slight modifications[36]. After 30 min of 6z (0.5–2 mg/kg, ip) or FLX (10 mg/kg, ip) or vehicle dosing, the mice were individually suspended by the tail to a horizontal bar (50-cm distance from the floor) using Scotch tape (approximately 1-cm distance from the tip of tail). Typically, the mice exhibited several escape-oriented behavior interspersed with temporally increasing bouts of immobility. The duration of immobility (s) during the 6-min test session was recorded.

Chronic study: chronic unpredictable stress

The CUS procedure was adopted, as previously described[37] with slight modifications. The model consisted of chronic exposure to variable unpredictable stressors, none of which was sufficient alone to elicit long-lasting effects. The mice in the CUS groups were subjected to different types of stressors (Table 1), whereas the mice in the normal control group were left undisturbed, except for the general housekeeping procedure. The CUS procedure was continued for four suc-

Table 1. Protocol for chronic unpredictable stress.

Day	Stressor	Duration of stress (h)
1	Restraint	2
2	Food deprivation	24
3	Exposure to foreign object	4
4	Exposure to empty bottle	6
5	Cage tilting at 45°	4
6	Predator (Wistar rat) exposure	2
7	Inversion of light and dark cycle	12
8	Exposure to cages with rat odor	6
9	Cage tilting at 45°	6
10	Isolation	24
11	Wet bedding	4
12	Water deprivation	24
13	Exposure to empty bottle	6
14	Food deprivation	24
15	Wet bedding	6
16	Restraint	2
17	Predator (Wistar rat) exposure	2
18	Exposure to cages with rat odor	4
19	Food deprivation	12
20	Isolation	24
21	Cage tilting at 45°	6
22	Food deprivation	12
23	Exposure to empty bottle	24
24	Inversion of light and dark cycle	12
25	Restraint	2
26	Wet bedding	4
27	Isolation	24
28	Cage tilting at 45°	6

cessive weeks with 6z (0.5, 1, and 2 $mg \cdot kg^{-1} \cdot d^{-1}$, *po*) or FLX (10 $mg \cdot kg^{-1} \cdot d^{-1}$, *po*) or vehicle administration during the last 2 weeks of the stress procedure. This procedure was followed by behavioral and biochemical assessments. The behavioral assays were performed 24 h after the last dosing to avoid the acute effects of the medication with one behavioral test performed on each day to avoid the residual effect of the earlier testing paradigm. Moreover, plasma and brain samples were collected 24 h after the last behavioral assay to eliminate the effect of acute stress on the biochemical parameters.

Forced swim test

FST was conducted, as previously described with slight modifications[38]. The mice were individually dropped into a plexiglass cylinder (height, 30 cm; diameter, 22.5 cm) filled with water to a depth of 15 cm and maintained at 23–25 °C. In this test, after an initial vigorous activity of 2 min, the mice acquired an immobile posture, which was characterized by motionless floating in the water and making only those movements necessary to keep the head above water. The duration of immobility (s) was recorded during the last 4 min of the 6-min test. The mice were subjected to a 15-min training session under similar conditions, 24 h before the test.

Sucrose preference test

The sucrose preference test was conducted, as previously described[39]. The test was conducted in the following three phases: phase 1, habituation; phase 2, sucrose preference baseline; and phase 3, sucrose preference testing. In phase 1, tap water in the homecage was replaced with 1% *w/v* sucrose in tap water for 24 h to habituate the mice to the novel solution. In phase 2, each mouse was transferred to a single cage and subsequently exposed to both tap water and sucrose solution for 3 days to attain the sucrose preference baseline. Sucrose preference was then determined by a two-bottle choice test using standard bottles, one filled with tap water and one filled with 1% sucrose solution, which were supplied to the mice for 24 h (phase 3). The locations of water and sucrose (left/right) were counterbalanced across the study. The tap water and sucrose solution intake was quantified by subtracting the final weight of bottles after the 24-h exposure period from their initial weight and averaged for 2 d. The preference was calculated as % preference=[(sucrose intake/total intake)×100].

Biochemical assays

Brain homogenate preparation

To assess oxidative brain damage, first, the mice were sacrificed; the brains were collected and immediately placed on ice and washed with sodium phosphate buffer (0.1 mol/L, pH 7.4). The brain samples were then homogenized in 10 volumes of sodium phosphate buffer (0.1 mol/L, pH 7.4) and centrifuged (Remi, cooling compufuge, CPR-24, India) at 13 523×*g* for 20 min. The pellets were discarded. The supernatants were collected and the parameters were measured. All of the biochemical measures were normalized to the protein content, with bovine serum albumin considered to be the standard[40].

Estimation of lipid peroxidation

Malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substance (TBARS) according to the reported method[41]. Briefly, 0.5 mL of brain homogenate and 0.5 mL of Tris-HCl were incubated at 37 °C for 2 h. After incubation, 1 mL of 10% trichloroacetic acid was added and centrifuged at 200×*g* for 10 min. To 1 mL of supernatant, 1 mL of 0.67% thiobarbituric acid was added and the tubes were kept in boiling water for 10 min. After cooling, 1 mL double distilled water was added and absorbance was measured at 532 nm (UV-1800 spectrophotometer, Shimadzu, Japan). The amount of lipid peroxidation products (TBARS) was quantified using an extinction coefficient of 1.56×10^5 $(mol/L)^{-1} \cdot cm^{-1}$ and expressed as nanomoles of MDA per milligram of protein.

Estimation of nitrite levels

Nitrite levels were estimated using the Greiss reagent, which served as an indicator of nitric oxide production[42]. A measure of 500 μL of the Greiss reagent (1:1 solution of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthaylamine diamine dihydrochloric acid in water) was added to 500 μL of brain homogenate, the mixture was incubated for 10 min at

room temperature in the dark, and absorbance was measured at 546 nm (UV-1800 spectrophotometer). The brain nitrite levels were calculated using a standard curve for sodium nitrite and were expressed as micromoles per milliliter.

Estimation of catalase (CAT) activity

The catalase activity was assayed using the standard method[43]. The assay mixture consisted of 1.95 mL phosphate buffer (0.05 mol/L, pH 7.0), 1.0 mL hydrogen peroxide (0.019 mol/L) and 0.05 mL brain homogenate (10%) in a final volume of 3.0 mL. The changes in absorbance were recorded at 240 nm. The catalase activity was calculated and expressed as micromoles of hydrogen peroxide consumed per minute per milligram of protein (U/mg protein).

Estimation of reduced glutathione (GSH) levels

Reduced glutathione in the brain was estimated according to the method described by Ellman[44]; 1 mL of supernatant was precipitated with 1 mL of 4% sulfosalicylic acid and cold digested at 4 °C for 1 h. The samples were centrifuged at 1200×*g* for 15 min at 4 °C. To 1 mL of supernatant, 2.7 mL of phosphate buffer (0.1 mol/L, pH 8) and 0.2 mL of 5,5-dithio-*bis* (2-nitrobenzoic acid) were added. The color developed was measured immediately at 412 nm (UV-1800 Spectrophotometer). The results are expressed as micromoles per milligram protein.

Plasma corticosterone estimation

The mice were decapitated, and blood was collected in clean centrifuge tubes containing disodium ethylenediaminetetraacetate (EDTA) as anticoagulant. The tubes were subsequently centrifuged at 13 523×*g* for 20 min at 4 °C. The plasma was separated and stored at -80 °C until the CORT estimations were performed. The CORT assay was performed using the method of Katyare and Pandya[45]. Plasma (1 mL) was treated with 0.2 mL of freshly prepared chloroform: methanol mixture (2:1 *v*/*v*), followed by extraction with 3 mL of chloroform. The chloroform extract was treated with 0.3 mL of sodium hydroxide (0.1 mol/L) and, subsequently, with 3 mL of 30 mol/L sulfuric acid. The tubes containing the sulfuric acid layer were kept in the dark for 30–60 min; thereafter, fluorescence measurements were performed in an SL-174-spectrofluorometer with excitation and emission wavelengths set at 472 nm and 533 nm, respectively. The plasma CORT contents are expressed as percentage with respect to the non-CUS group (taking the non-CUS group values as 100%).

Statistical analysis

The data are expressed as the mean±standard error (SEM). The statistical analysis was performed using GraphPad Prism version 3.0. The data in the acute study were analyzed using one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's test. All of the data in the chronic study were statistically analyzed using one-way ANOVA followed by *post hoc* Tukey's multiple comparison test. $P<0.05$ was considered to be statistically significant.

Results

5-HT_3 receptor antagonistic activity of 6z

The ligand, 6z, was synthesized as 5-HT_3 receptor antagonist; therefore, an examination of the antagonist affinities of 6z at 5-HT_3 receptors in the guinea pig ileum was conducted. The study confirmed the 5-HT_3 antagonistic activity of the compound as indicated by the pA_2 value (7.4). The data indicate that 6z affinity for guinea-pig 5-HT_3 receptors is more than that observed for ondansetron (pA_2 value=6.9).

Dose response study

The effects of different doses of 6z were observed for stimulation of the baseline locomotor activity. There was no significant change in the SLA of mice [$F_{(5,36)}$=1.52, P=0.2078]. Acute treatment with 6z (0.5–2 mg/kg, ip) did not produce significant effects on the SLA (*post hoc* Dunnett's test, $P>0.05$ *vs* control group), whereas 6z at 4 mg/kg produced a significant increase in the SLA in mice (*post hoc* Dunnett's test, $P<0.05$ *vs* control group). Therefore, the antidepressant-like effects of the agent 6z in acute and chronic models were evaluated using 0.5–2 mg/kg dose ranges. Similarly, the positive control, FLX (10 mg/kg, ip) did not affect the SLA in mice (Figure 2) (*post hoc* Dunnett's test, $P>0.05$ *vs* control group).

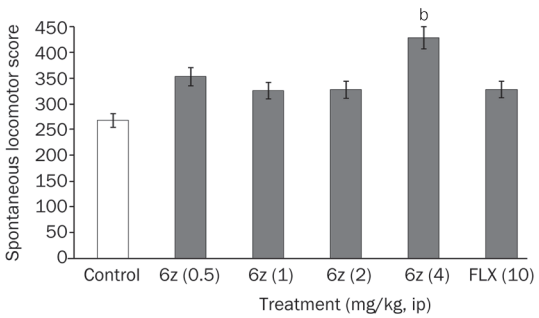

Figure 2. Effects of 6z and FLX on spontaneous locomotor activity in mice. The columns represent mean values of spontaneous locomotor scores, while error bars show SEM. The results from *post hoc* Dunnett's test are indicated in the figure. $^bP<0.05$ as compared to the control group, n=7 mice/group.

Behavioral assays

Acute study: tail suspension test

Figure 3 shows the influence of acute treatment of mice with 6z (0.5–2 mg/kg, ip) and FLX (10 mg/kg, ip) on the duration of immobility in the TST. There was a significant effect of 6z and FLX on the duration of immobility in mice during the TST [$F_{(4,30)}$=4.43, P=0.0062]. Acute treatment with 6z (0.5–2 mg/kg) significantly decreased the duration of immobility (s) compared with the vehicle-treated group [*post hoc* Dunnett's test, $P<0.05$ for 6z (0.5 mg/kg) and $P<0.01$ for 6z (1–2 mg/kg) *vs* control]. Similarly, the positive control, FLX (10 mg/kg), decreased the duration of immobility in mice during the TST (*post hoc* Dunnett's test, $P<0.01$).

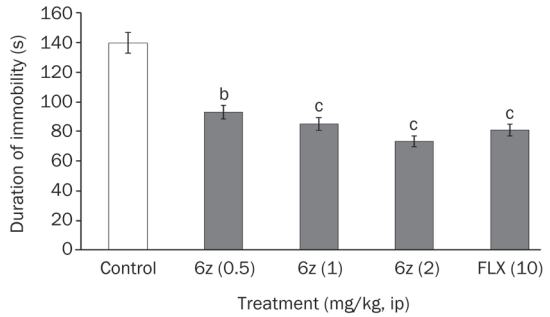

Figure 3. Effects of 6z and FLX on duration of immobility during TST in mice. The columns represent mean duration of immobility(s) values, while error bars show SEM. The results from *post hoc* Dunnett's test are indicated in the figure. $^{b}P<0.05$, $^{c}P<0.01$ as compared to the control group. $n=7$ mice/group.

Chronic study

Forced swim test

Figure 4A shows the effects of 6z and FLX (10 mg/kg, *po*) on the depressive-like behavior induced by CUS in mice, as measured by FST. One-way ANOVA revealed a significant difference among the groups [$F_{(5,36)}=4.02$, $P=0.0053$]. The *post hoc* analysis indicated that CUS produced a significant increase in the duration of immobility in mice compared with non-stressed mice (Tukey's multiple comparison test, $P<0.05$ *vs* non-CUS mice). Chronic treatment with 6z (0.5–2 mg/kg, *po*) significantly reversed the elevated duration of immobility in chronically stressed mice [Tukey's multiple comparison test, $P<0.05$ for 6z (0.5 and 2 mg/kg) and $P<0.01$ for 6z (1 mg/kg) *vs* CUS mice]. Furthermore, the repeated treatment with FLX significantly reduced the duration of immobility in CUS mice (Tukey's multiple comparison test, $P< 0.01$ *vs* CUS mice).

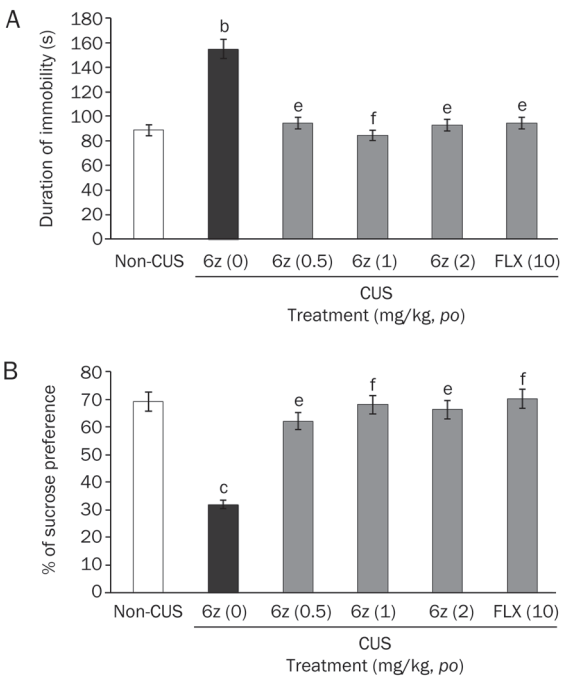

Figure 4. Effects of 6z and FLX on (A) duration of immobility during FST and (B) % of sucrose preference in CUS mice. The columns represent mean values, while error bars show SEM. The results from *post hoc* Tukey's Multiple Comparison test are indicated in the figure. $^{b}P<0.05$, $^{c}P<0.01$ as compared to the unstressed control group (non-CUS). $^{e}P<0.05$, $^{f}P<0.01$ as compared to stressed control mice (CUS) respectively. $n=7$ mice/group.

Sucrose preference test

Figure 4B shows the influence of 6z and FLX on the % of preference of sucrose consumption over drinking water in mice. One-way ANOVA revealed a significant difference among the groups [$F_{(5,36)}=4.55$, $P=0.0026$]. *Post hoc* analysis demonstrated a pronounced decrease in the % of sucrose preference in stressed mice compared with unstressed mice [Tukey's multiple comparison test, $P<0.01$ *vs* non-CUS mice]. Chronic treatment with 6z (0.5–2 mg/kg) significantly increased the % of sucrose preference in stressed mice [Tukey's multiple comparison test, $P<0.05$ for 6z (0.5 and 2 mg/kg) and $P<0.01$ for 6z (1 mg/kg) *vs* CUS mice]. Moreover, the positive control FLX (10 mg/kg) elicited a significant increase in the % of sucrose preference in CUS mice (Tukey's multiple comparison test, $P<0.01$ *vs* CUS mice).

Biochemical assays

Lipid peroxidation

As depicted in Figure 5A, there was a significant difference in the brain TBARS level (a measure of lipid peroxidation product) among the groups [$F_{(5,36)}=4.21$, $P=0.0041$]. Exposure to CUS significantly increased the brain TBARS level in mice (Tukey's multiple comparison test, $P<0.01$ *vs* non-CUS). Chronic treatment with 6z (0.5–2 mg/kg) produced a significant reduction in TBARS level in the brain of CUS mice (Tukey's multiple comparison test, $P<0.05$ *vs* CUS). Similarly, FLX (10 mg/kg) reduced TBARS level in the brain of CUS mice (Tukey's multiple comparison test, $P<0.01$ *vs* CUS).

Nitrite level

There was a significant change in the brain nitrite level among the groups [$F_{(5,36)}=3.65$, $P=0.009$]. The one-way ANOVA statistical analysis revealed that chronic stress produced a significant increase in the nitrite level in the brain of mice subjected to CUS (Tukey's multiple comparison test, $P<0.05$ *vs* non-CUS), whereas the repeated administration of 6z (1–2 mg/kg) reversed CUS-induced altered brain nitrite level (Tukey's multiple comparison test, $P<0.05$ *vs* CUS) as shown in Figure 5B. However, 6z at 0.5 mg/kg had no effect on the brain nitrite level in stressed mice (Tukey's multiple comparison test, $P>0.05$ *vs* CUS). Furthermore, the positive control FLX (10 mg/kg) significantly produced a decrease in nitrite level in the brain of mice subjected to CUS (Tukey's multiple comparison test, $P<0.05$ *vs* CUS).

Catalase activity

As shown in Figure 5C, catalase activity was significantly altered among the groups subjected to different treatments

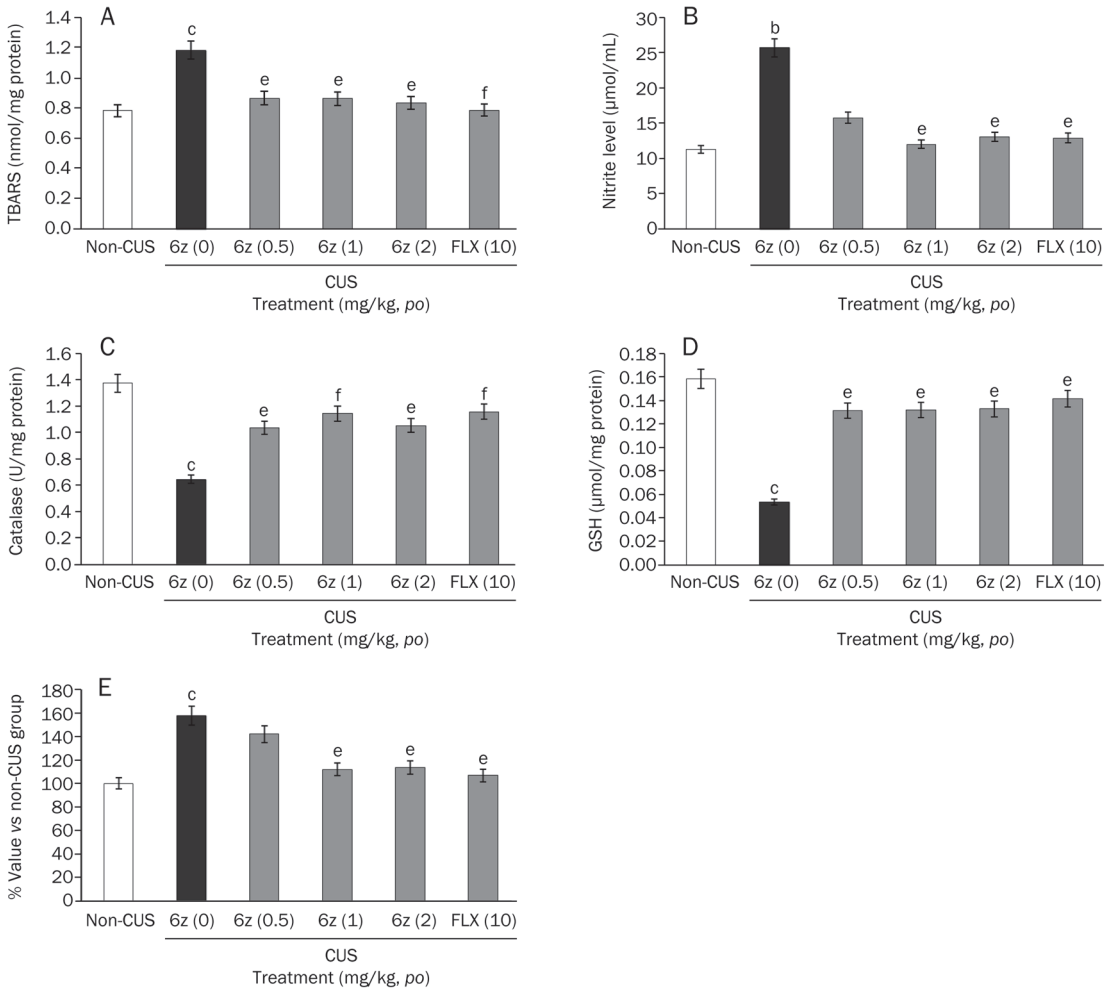

Figure 5. Effects of 6z and FLX on (A) the brain TBARS level, (B) the brain nitrite levels, (C) the brain catalase activity, (D) the brain reduced glutathione (GSH), (E) the plasma corticosterone (CORT) levels in mice exposed to chronic stress. The columns represent values, while error bars show SEM. The results from *post hoc* Tukey's Multiple Comparison test are indicated in the figure. $^{b}P<0.05$, $^{c}P<0.01$ as compared to the unstressed control group (non-CUS). $^{e}P<0.05$, $^{f}P<0.01$ as compared to stressed control mice (CUS), respectively. $n=7$ mice/group.

[$F_{(5,36)}$=6.95, P=0.0001]. Catalase activity was significantly reduced in the brain of mice submitted to the CUS procedure compared with non-CUS mice (Tukey's multiple comparison test, $P<0.05$ *vs* CUS). Chronic treatment with 6z (0.5–2 mg/kg, *po*) in CUS mice displayed significantly increased catalase activity in the brain (Tukey's multiple comparison test, $P<0.05$ *vs* CUS mice for 6z at 0.5 and 2 mg/kg and $P<0.01$ for 6z at 1 mg/kg). Additionally, the positive control FLX (10 mg/kg, *po*) elicited a significant increase in the catalase activity in the brain of CUS mice (Tukey's multiple comparison test, $P<0.01$ *vs* CUS group).

Reduced glutathione

There was a significant difference in the brain GSH levels among the groups [$F_{(5,36)}$=6.17, P=0.0024]. The brain GSH levels were significantly depleted in stressed mice compared to normal control mice, as illustrated in Figure 5D (Tukey's multiple comparison test, $P<0.01$ *vs* non-CUS mice). This reduction, induced by CUS, was significantly blunted by the chronic administration of 6z (0.5–2 mg/kg, *po*) and FLX (10 mg/kg, *po*) (Tukey's multiple comparison test, $P<0.05$ *vs* CUS group).

Plasma CORT assay

Regarding the plasma CORT levels in mice, one-way ANOVA revealed a significant difference among the groups subjected to different treatments [$F_{(5,36)}$=5.01, P=0.0012]. Chronic stress produced a pronounced increase in the % of plasma CORT levels in mice (Tukey's multiple comparison test, $P<0.01$ *vs* non-CUS group), as shown in Figure 5E. The administration of 6z (1–2 mg/kg, *po*) and FLX (10 mg/kg, *po*) for 2 weeks abolished the CUS-induced elevated % of plasma CORT levels (Tukey's multiple comparison test, $P<0.05$ *vs* CUS group). However, the administration of 6z at lower dose of 0.5 mg/kg had no effect on the % of plasma CORT levels in stressed mice (Tukey's multiple comparison test, $P>0.05$ *vs* CUS group).

Discussion

The current inconsistent pharmacotherapy and consistent increase in the prevalence of depressive disorders necessitate the development of compounds with novel targets, which may provide better therapeutic efficacy[1, 3]. The present study investigated the antidepressant-like effects of 6z, a novel 5-HT_3 receptor antagonist, in acute and chronic murine models of depression. Preliminary 5-HT_3 receptor antagonistic activity of 6z (in the form of pA_2), which was evaluated using guinea pig ileum LMMP model, was higher than the standard agent, ondansetron, indicating the potential 5-HT_3 receptor affinity and antagonistic action of 6z[25]. The acute treatment with 6z (0.5–2 mg/kg, ip) resulted in an antidepressant-like effect in mice, which was indicated by the decreased duration of immobility during TST. In agreement with the acute study, the chronic administration of 6z (0.5–2 mg/kg, *po*) reversed the depressive-like behavior induced by a 4-week chronic stress protocol in mice. It also demonstrated the antidepressant-like behavior of 6z in the chronic stress model of depression. Additionally, 6z (0.5–2 mg/kg) abolished chronic stress-induced biochemical derangements, such as increased brain oxidative stress and plasma CORT level, a putative marker of hyperactive HPA-axis function in CUS mice, demonstrating the likely mechanism of action involved in the postulated effect of the test compound.

The TST is one of the most frequently reported models for assessing antidepressant-like effects of compounds. It is based on the principle that mice exposed to the short-term inescapable stress of being suspended by their tail develop an immobile posture, which reflects a state of helplessness, one of the core symptoms of depression observed in humans[14, 36]. It has been reported that medications with antidepressant-like effects decrease immobility time in mice[1, 14]. In the current study, acute dosing with 6z (0.5–2 mg/kg, ip) produced a significant decrease in the duration of immobility, demonstrating antidepressant-like behavior. Similarly, FLX, a conventional antidepressant used as a positive control, produced antidepressant-like behavior with a significant decrease in the duration of immobility in mice. This finding is in agreement with previous reports demonstrating that numerous antidepressants as well as 5-HT_3 receptor antagonists produce antidepressant-like activity and reduce the duration of immobility in TST[7, 37].

However, it has been found that medications with pyschostimulant effects also decrease the duration of immobility in TST[38]. Therefore, the effects of 6z (0.5–2 mg/kg) on the SLA of mice was evaluated. 6z, similar to FLX, had no effect on the SLA, which clarified that the behavioral effect of 6z (0.5–2 mg/kg) in TST was not merely due to psychomotor stimulation.

Although acute behavioral studies substantially demonstrate the pharmacological activity of the compounds, the tested doses do not correspond with the clinical time course of their action. Therefore, the antidepressant-like effect of 6z, as observed in the acute model, was also evaluated in the chronic murine model of depression.

CUS is one of the most promising and valuable tools for studying depressive behavior in animals and for screening antidepressant-like effects of novel compounds. Exposure to uncontrollable stressors in an unpredictable manner develops a state of depressive-like behavior in rodents, which has been found to simulate stress-induced pathophysiological conditions in depressed patients[15]. However, the duration of exposure, variability and unpredictability of stressors are critical factors in the development of depressive-like behavior[18, 39]. Additionally, CUS has been reported to have the high predictive, face and constructive validities that are required for a model to be valid in psychiatric disorders[18].

To evaluate behavioral effects as a consequence of chronic stress in mice, the forced swim test has been widely utilized[40]. Stressed mice subjected to the FST represent increased duration of immobility, which reflects behavioral despair, although treatment with chronic antidepressants can reverse the condition[27]. In agreement with previous reports, the current study showed a pronounced depressive behavior induced by CUS, indicated by increased duration of immobility in mice. Additionally, with the chronic administration of positive control, FLX abolished the behavioral despair in CUS mice during the FST, which suggested a high predictive validity of the model[18]. Interestingly, 6z (0.5–2 mg/kg) chronic treatment (in accordance with its acute effect) reversed the CUS-induced increased immobility time, which suggested the antidepressant-like behavior of the test compound. Furthermore, the result corroborates the previous findings that 5-HT_3 receptor antagonists can reverse the chronic stress-induced behavioral deficits in FST[17].

Sucrose preference test is a valid and useful marker of chronic stress-induced behavioral impairments in animals[18]. It is considered a putative indicator of anhedonia (the loss of pleasure), one of two symptoms required for diagnosing a major depressive episode in humans[36]. It is well reported that exposure to chronic stress damages the nerve cells in neuronal reward systems, including serotonergic and dopaminergic (DA) systems. 5-HT and DA systems are involved in the regulation of reward and behavior, and impairment in these systems leads to the loss of ability to experience pleasure and reward activities[41]. Previous reports have shown that chronic stress develops reward-related behavioral derangements, which can be reversed by chronic treatment with antidepressants including 5-HT_3 antagonists[16, 17]. In the present study, CUS elicited significant reduction in reward-related behavior (anhedonia) indicated by a decrease in the % of sucrose preference in mice. Similar to the positive control FLX, 6z (0.5–2 mg/kg) attenuated chronic stress-induced anhedonia in mice. Therefore, it may be suggested that the test compound 6z may have modulatory effects on the neuronal reward systems (5-HT and DA). However, additional studies are warranted to affirm this hypothesis. Moreover, the present study estimated the modulation of the brain oxidative stress and HPA-axis functions as the likely mechanism of action of 6z.

Increased oxidative stress has been associated with chronic stress that leads to severe neuronal injury and functional impairments. Chronic studies suggested that oxidative brain

damage may play a role in the pathogenesis of depression[21]. In the present investigation, mice exposed to CUS for 4 weeks exhibited increased brain oxidative stress, as evidenced by elevated pro-oxidant markers, such as lipid peroxidation and nitrite levels, and reduced antioxidant enzyme (catalase and reduced glutathione) activity, which could be correlated with the behavioral deficits observed during the FST (elevated duration of immobility) and sucrose preference test (decreased % of sucrose preference). This observation is in accordance with a previous report, which showed that 21 days of exposure to unpredictable stressors resulted in increased lipid peroxidation in the brain[39]. Additionally, consistent with the results of the present study, Lucca *et al*[19] demonstrated an increase in TBARS level in discrete areas of the rat brain subjected to a 40-d chronic stress protocol. Furthermore, clinical studies investigating biochemical parameters in depressed patients have demonstrated increased oxidative stress markers in the brain[21]. Chronic treatment with 6z, similar to FLX, reversed the stress-induced oxidative load in the mice brain as indicated by reduced lipid peroxidation, nitrite level and elevated antioxidant enzyme (such as reduced glutathione and catalase) functions compared with stressed control mice. The results are supported by a recent study that 5-HT_3 antagonists, in addition to several clinical antidepressants, abolish stress-induced increased brain oxidative stress[17, 21].

Although experimental studies and clinical reports support the idea that stress-induced depressive-like behavior is associated with increased brain oxidative stress[19], the molecular mechanisms mediating the potential relationship are not completely understood. However, a link between chronic stress-induced oxidative brain damage and increased levels of calcium ions at the cerebrocortical nerve terminal in rodents has been demonstrated to be involved in the etiopathogenesis of depression[43]. Pathologically, high levels of calcium ions enter the nerve cell and stimulate the production of ROS[20, 44], which may cause direct damage to cellular proteins, DNA and lipids, and consequently lead to the loss of cell membrane fluidity and abnormalities of monoaminergic receptor function[20]. The stimulation of 5-HT_3 receptors has been characterized by an increased concentration of calcium ions at the brain nerve terminals, which is potentiated by agonist activation of 5-HT_3 receptors[45]. Considering the likelihood of 6z in antagonizing the 5-HT_3 receptors mediated the release of calcium ions, one could hypothesize the involvement of the pathway in reversing the chronic stress-induced brain oxidative damage and hence the antidepressant-like effects of the test compound[46]. However, additional studies are necessary to confirm such a hypothesis.

Additionally, hyperactive HPA-axis function is reported to be involved in the pathogenesis of chronic stress-induced depressive-like behavior[22]. The stimulation of HPA-axis results in elevated glucocorticoid-receptor functions, which can be characterized by enhanced circulating levels of glucocorticoids[47]. The excessive glucocorticoids may lead to neurocellular damage in several regions of the brain, which are involved in maintaining mood and behavioral activity[47, 48]. This may be an important mechanism of neuropsychological impairment observed in depressed patients[49]. In preclinical findings, chronic stress has been reported to cause HPA-axis hyperactivity and to increase glucocorticoid levels[50]. Accordingly, in the present investigation, mice exposed to CUS resulted in hyperactivity of the HPA-axis, as evidenced by an enhanced % of plasma CORT levels. The administration of 6z (1–2 mg/kg) and fluoxetine significantly reduced the % of plasma CORT levels in stressed mice. Supporting the present findings, consistent evidence of the reversal of stress-induced HPA-axis hyperactivity by several antidepressants has been shown[23]. The reason for this effect of antidepressants (such as fluoxetine) is not known; however, antidepressant treatment may normalize HPA-axis functions via an indirect effect of actions on 5-HT system, given the multiple interactions between 5-HT and the HPA-axis[49].

Conclusion

Altogether, the findings in the present study target potential antidepressant-like effects of 6z, a novel 5-HT_3 receptor antagonist, in acute and chronic murine models of depression because 6z decreased the duration of immobility in the acute TST testing paradigm and abolished CUS-induced two parameters of depressive-like behavior, *ie*, the increased duration of immobility during the FST and anhedonia in the sucrose preference test, which are the putative indices of major depressive symptoms observed in humans. Furthermore, 6z improved the brain antioxidant system in parallel with behavioral changes. 6z normalized stress-induced HPA-axis hyperactivity. It suggested the multi-functional mechanism of action of the test compound. The behavioral and biochemical evidence obtained for 6z are comparable to those obtained for FLX. These findings are particularly interesting because 6z exhibited antidepressant-like effects in acute administration, which conforms with the chronic treatment (required for the clinical time course of action). Thus, considering that CUS model is closely related to changes that occur in depressed patients and that current pharmacotherapy exists with therapeutic inadequacy (in terms of intolerance and unresponsiveness), 6z may be further investigated as a novel agent for improving the therapeutics of depression.

Author contribution

Deepali GUPTA designed the experiment and prepared the manuscript draft. Deepali GUPTA and Yeshwant KURHE performed the evaluation of 6z in the CUS model for behavioral parameters in the FST and sucrose preference test. Visakh PRABHAKAR and Prateek KANADE evaluated 6z activity in the TST and determined oxidative stress parameters. Deepali GUPTA and Yeshwant KURHE performed plasma CORT estimation. The synthesis and spectral analysis of 6z were conducted by Devadoss THANGARAJ. Mahesh RADHAKRISHNAN contributed to the experiment design and manuscript edition. All of the authors contributed to the manuscript and approved the final manuscript as presented.

Supplementary information

Supplementary figure is available at the Acta Pharmacologica Sinica website.

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