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Anxiolytic-like effect of chalcone N-{4'[(2*E*)-3-(3-nitrophenyl)-1-(phenyl)prop-2-en-1-one]} acetamide on adult zebrafish (*Danio rerio*): Involvement of the 5-HT system

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ABSTRACT

The action of anxiolytic compounds that act on selective serotonin receptors (SSRIs) have been scarcely evaluated. Serotonergic drugs have been shown to be effective in treating anxiety without presenting adverse effects as benzodiazepines. However, the anxiolytic effects take days to occur. This study aimed to evaluate the anxiolytic effect of the synthetic chalcone, 4'-[(2*E*) -3- (3-nitrophenyl) -1- (phenyl) prop-2-en-1-one] acetamide (PAAMNBA), and its possible mechanism of action in adult zebrafish (*Danio rerio*). PAAMNBA was synthesized with a yield of 51.3% and its chemical structure was determined by ^{1}H and ^{13}C NMR. Initially, PAAPMNBA was intraperitoneally administered to zebrafish (n = 6/group) at doses of 4, 12, or 40 mg/kg, and the animals were subsequently subjected to acute and open field toxicity tests. PAAMNBA was administered to the other groups (n = 6/group) for analyzing its effect in the light and dark test. The involvement of the serotonergic (5HT) system was also evaluated using 5-HTR 1, 5-HTR 2A/2C, and 5-HTR 3A/3B receptor antagonists, namely, pizotifeo, granizetron, and ciproeptadina, respectively. Molecular coupling was performed using the 5-HT1 receptor. PAAMNBA was found to be non-toxic, reduced the locomotor activity, and had an anxiolytic effect in adult zebrafish. The effect was reduced by pretreatment with pizotifene and was not reversed by treatment with granizetron and cyproeptadine. A previous *in vivo* molecular coupling study indicated that chalcones interact with the 5-HT1 receptor. The results suggested that the chalcone, PAAPMNBA, has anxiolytic activity, that is mediated by the serotonergic system via the 5-HT1 receptor. The interaction of PAAPMNBA with the 5-HT1 receptor was confirmed by molecular docking studies.

1. Introduction

Anxiety and depression are considered to be the two most prevalent psychiatric disorders. The classical anxiolytic drugs and antidepressants, including benzodiazepines, monoamine oxidase

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inhibitors, tricyclic antidepressants, selective serotonin reuptake inhibitors, norepinephrine, and specific noradrenergic and serotonergic antidepressants, are widely used in clinical practice for treating such psychiatric disorders. However, treatment with the aforementioned drugs can also have undesirable side effects, including cardiovascular toxicity, sexual dysfunction, weight gain, and drug interactions [1–3].

Buspirone is a serotonergic drug that has been shown to be effective in treating anxiety without having the observed adverse effects. However, the anxiolytic effects appear only after 3–4 weeks of administration [4,5]. Therefore, there is an urgent need to develop effective anxiolytic and antidepressant therapies that are devoid of side effects. Chalcones belong to the flavonoid family, and their structure comprises two aromatic rings linked by a carbonyl system comprising three α,β-unsaturated carbons [6]. The derivatives of natural and synthetic chalcones have been demonstrated to have anxiolytic activity [7].

We have previously identified and reported the anxiolytic effect of the synthetic chalcone, N-(4′-[(2*E*)-3-(4-fluorphenyl)-1-(phenyl) prop-2-en-1-one] acetamide (PAAPFBA), in adult zebrafish [8]. The zebrafish model is rapidly becoming an important model organism in behavioral neuroscience as it offers numerous benefits over mammalian models, including rapid external development, small size, and low cost [9]. This organism shares genetic homology with mammals and has conserved molecular and cellular mechanisms [10]. Additionally, zebrafishes possess the major neurotransmitter systems, including GABA, glutamate, dopamine (DA), and serotonin (5-HT), among others [11,12]. This study therefore aimed to evaluate the anxiolytic effect of the synthetic chalcone, N-{4'- [(2*E*)-3-(3-nitrophenyl)-1- (phenyl) prop-2-en-1-one]} acetamide (PAAMNBA), and its possible mechanism of action in adult zebrafish (*Danio rerio*).

2. Materials and methods

2.1. Drogas e reagentes

The following reagents and drugs were used in the study: granisetron hydrochloride (Corepharma/Inglaterra-Mx), pizotifen maleate (Central Manipulation Pharmacy/Brasil-SP), fluoxetine (Eli Lilly/EUA-IN), Cyproheptadine (Evidence Soluções Farmacêuticas/ Brasil- CE) and Diazepam (Sigma-Aldrich/EUA-MO).

2.2. Synthesis and chemical characterization of chalcone PAAPMNBA

The chalcone PAAMNBA was synthesized through the aldol condensation reaction of Claisen-Schmidt in basic medium using *p*-aminoacetophenone and *m*-Nitrobenzaldehyde, followed by the acetylation reaction with acetic anhydride in buffered medium (acetic acid/sodium acetate) at pH 4.5 under magnetic stirring [13], Scheme 1. The Chemical Structure of PAAMNBA was determined by ^{1}H and ^{13}C NMR, FT-IR and CG-MS.

2.3. Adult zebrafish (D. rerio) (aZF)

Wild adult zebrafish (60–90 days old; 0.4 ± 0.1 g, 3.5 ± 0.5 cm), of both genders, were obtained from a commercial supplier (Fortaleza, CE). Groups of animals (n = 50) were acclimatized for 24 h in a 10 L glass tank (30 × 15 × 20 cm) containing dechlorinated tap water (ProtecPlus®) and a submersible filter and air pump at 25 °C and pH 7.0, under a circadian cycle of 14:10 h (light/dark). Animals received *ad libitum* feed 24 h prior to the experiments. After the experiments, the animals were euthanized by immersion in ice water (2–4 °C) for 10 min until loss of opercular movements occurred. All experimental procedures were approved by the Animal Research Ethics Committee of the State University of Ceará (CEUA-UECE # 7210149/2016).

2.4. Acute toxicity to adult zebrafish

The study was carried out based on the methodology with adaptations [14]. As an adaptation of the method, aZF (n = 6/group) were treated intraperitoneally (*i.p.*) with 20 μL of the PAAPMNBA (4.0 ou 12 ou 40 mg/kg). As negative control, 3% Dimethyl sulfoxide (DMSO) (20 μl, *i.p.*) was used. After 24, 48, 72 and 96 h, the values obtained regarding the number of dead aZF were submitted to statistical analysis, estimating the lethal dose to kill 50% (LD_{50}) of aZF utilizing the *Trimmed Spearman*-Karber method with 95% confidence intervals.

2.5. Evaluation of locomotor activity

The Open Field Test was used to evaluate whether or not there was alteration of the motor coordination in the animals aZF (n = 6/group) were treated intraperitoneally (*i.p.*) with PAAPMNBA (4.0 ou 12 ou 40 mg/kg) or vehicle (DMSO 3%) or diazepam (DZP 4.0 mg/kg). After 30 min of the treatments, the animals were added to Petri dishes containing the same water of the aquarium, marked with four quadrants and the locomotor activity was analyzed by counting the number of line crossings, during 5 min. Animals that did not receive treatments (Naïve) were considered as baseline (100% of locomotor activity) [15].

2.6. Test light & dark

The anxiolytic-like effect of PAAPMNBA was investigated through the Light & Dark Test [16]. The animals (n = 6/group) were treated intraperitoneally with PAAPMNBA (4.0 ou 12 ou 40 mg/kg) or vehicle (Control, 3% DMSO, 20 μL) or diazepam (Dzp; 4.0 mg/kg). An untreated group (Naïve) was included. After 30 min of the treatments, the animals were added to the light zone of the glass aquarium (30 × 15 × 20 cm), which was divided into light and dark zones, with drug-free water. The anxiolytic-like effect was characterized by the presence of the animals in the light zone, during 5 min of analysis.

2.7. Involvement of the serotonergic system (5-HT)

Groups of animals (n = 6) received cyproheptadine (5-HT_{2A} antagonist) or pizotifen (5-HT_1 antagonist and 5-$HT_{2A/2C}$) orally, either at a dose of 32 mg/kg or granisetron (5-$HT_{3A/3B}$ antagonist, 20 mg/kg, orally) 30 min before PAAPMNBA (4.0 mg/kg; *i.p.*) or fluoxetine (Flx; 0.05 mg/kg, *i.p.*). Subsequently, the Light & Dark Test was performed as cited previously.

2.8. Docking

The interaction between the receptor, 5-HT1, and the chalcone, PAAPMNBA, was analyzed by molecular coupling simulation, which involves the use of computational software with a bimolecular coupling algorithm, with the aim of forming a stable complex. The three-dimensional structure of 5-HTR 1 was retrieved from the Protein Data Bank (PDB ID: 3J5P). The structure was obtained by crystallography, with a resolution of 3.9 Å [17]. The 3D structure of the chalcone was modeled from the chemical structure (Fig. 1A and B) with Avogadro software version 1.1.1, while considering its stereochemistry. The fitting was performed using HEX software, version 8.0.0 [18]. The software performed automatic fittings based on the energy of interaction between PAAPMNBA and each of the

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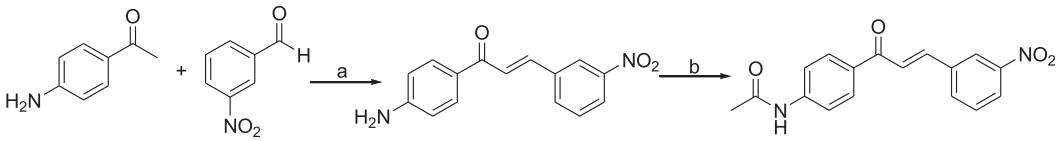

Scheme 1. Preparation of chalcone a) NaOH 50% w/v, ethanol, r.t., 48 h. b) $(CH_3CO)_2$, (AcOH/AcONa), r.t.

possible interaction sites in the 5-HT1 receptor, which is also known as the serotonin receptor. The clusters that were obtained were subsequently analyzed using PyMol version 1.4.7, which allowed a detailed investigation of the complexes [19].

2.9. Statistical analysis

Results were expressed as mean ± standard deviation of the mean for *in vitro* tests (n = 3) as well as mean ± standard error of the mean for *in vivo* tests (n = 6/group). After confirming the distribution of normality and homogeneity of data, differences between the groups were submitted to analysis of variance (one-way ANOVA), followed by Tukey test, using the software GraphPad Prism v. 7.0. The level of statistical significance was considered at 5% ($p < 0.05$).

3. Results

3.1. Síntese e caracterização da chalcona PAAPMNBA

The chalcone N-{4'-[(2*E*)-3-(3-nitrophenyl)-1-(phenyl)prop-2-en-1-one]}acetamide) (PAAPMNBA) (Fig. 1A) was synthesized and yield was 51.29% orange solid. Orange solid (Yield: 51,29%), m.p. 198−198,3 °C; FT-IR (KBr, ν_{cm}^{-1}): 3330, 1610, 1660, 1580, 1340, 970. ^{1}H NMR (CD_3SOCD_3, 300 MHz): δ 8.17−7.75 (m, H-4, H-5), 8,17 (d, H-6, J = 10.53 Hz), 7.96 (d, H-2'/H-6', J = 7.41 Hz), 6.63 (d, H-3'/H-5', J = 7.32 Hz), 8.00 (d, H-α, J = 17.04), 8.71 (d, H-β, J = 14.88 Hz), 2.09 (CH_3). NMR de ^{13}C (CD_3SOCD_3, 75 MHz): δ C-1134.9, C-2122.8, C-3148.4, C-4122.6, C-5130.1, C-6134.7, C-1′ 137.1, C-2'/C-6′ 131.4, C-3'/5′ 112.9, C-4′ 144.0, C-α 122.9, C-β 144.0, C═O 187.4, N−C═O 169.2, CH_3 24.2. HRESIMS, *m*/*z*: 310.1445($C_{19}H_{19}NO_3$) $[M + H]^+$ (calcd. 310.1443).

3.2. Acute toxicity (96 h)

The chalcone PAAPMNBA was non-toxic to adult zebrafish until 96 h of analysis (DL_{50} > 40 mg/kg).

3.3. Open field test

The PAAPMNBA chalcone [***p < 0.001 (4.0 mg/kg); ****p < 0.0001 (12 and 40 mg/kg)] and diazepam [****p < 0.0001 (40 mg/kg)] significantly decreased the locomotor activity of the adult zebrafish compared to the groups controls (naive and vehicle) (Fig. 2A).

3.4. Anxiolytic evaluation (Light & Dark Test)

PAAPMNBA (4.0 or 12 or 40 mg/kg) promoted (****p < 0.0001 vs naive or vehicle) anxiolytic effect in aZF in the Claro & Escuro test (Fig. 2B). This effect was significantly similar to the effect of diazepam (DZP; 40 mg/kg; *i.p.*).

3.5. Involvement of the serotonergic system (5-HT)

3.5.1. Involvement of the 5-HTR3A/3B system

Granisetron did not reduce the anxiolytic effect of chalcone PAAPMNBA (4.0 mg/Kg, *i.p.*). However, granisetron reduced ($^{\#\#\#\#}$p < 0.0001 vs. Flx) the anxiolytic effect of fluoxetine (Flx; 0.05 mg/Kg; *i.p.*) (Fig. 3A).

3.5.2. Involvement of the 5-HTR$_{2A}$ system

Cyproeptadine did not reduce the anxiolytic effect of chalcone PAAPMNBA (4.0 mg/kg, *i.p.*). Contudo, cyproeptadine reduced ($^{\#\#\#\#}$p < 0,0001 *vs.* Flx) the anxiolytic effect of fluoxetine (Flx; 0.05 mg/kg; *i.p.*) (Fig. 3B).

3.5.3. Involvement of the 5-HTR 1 and 5-HTR2A/2C systems

Pizotifene reduced ($^{\#\#\#\#}$p < 0.0001 vs. PAAPMNBA or Flx) the anxiolytic effect of chalcone PAAPMNBA (4.0 mg/Kg, *i.p.*) and fluoxetine (Flx; 0.05 mg/Kg; *i.p.*) (Fig. 3C).

3.6. Molecular docking

Nine chemical bonds (1.7−4.0 Å) and high conformational stability were found between the amino acid residues Thr203, Asp204, Asn202, Arg188, Val200, Val201, Val233, Pro338 and Met337 of the alpha-helix of the 5-HTR1 receptor and the chalcone PAAPMNBA (Fig. 1B).

4. Discussion

The ^{13}C NMR spectrum of the PAAPMNBA showed the characteristic signals of a ketone (C = O) conjugated to an α,β-unsaturated system at δ_C = 187.4 ppm. Signals for Cα and Cβ were also obtained at 122.9 ppm and 144 ppm, respectively, which characterize the enona system. The presence of the acetyl group was confirmed by the absorption of the N−C═O group at δ_C = 169.2 ppm, and the methyl group was confirmed by the signal at δ_C = 24.2 ppm in. The ^{1}H NMR spectrum revealed signals of ^{1}H, corresponding to C-sp2 that formed a doublet with coupling values of 17.04 Hz for Hα and 14.88 Hz for Hβ, thus confirming the trans stereochemistry of the

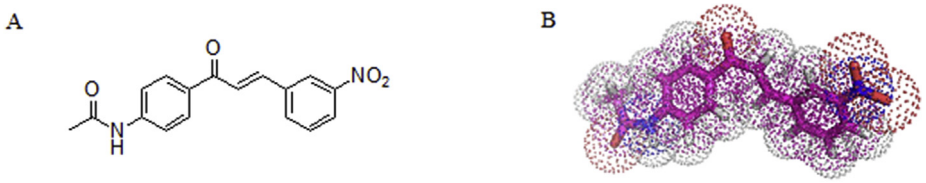

Fig. 1. Chemical structure (A) and Three-dimensional structure (B) of the chalcone PAAPMNBA.

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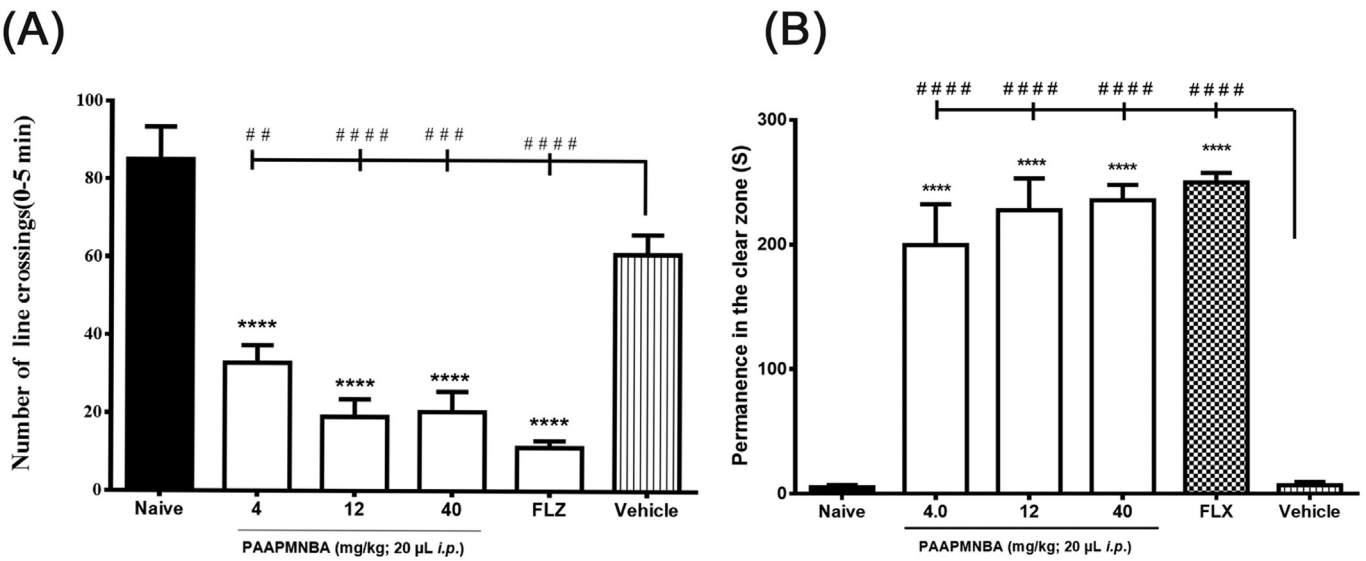

Fig. 2. Effect under the locomotor behavior of zebrafish (*Danio rerio*) adult in the Open Field Test (0−5 min) (A) and ansiolitic-like dffect on adult zebrafish (*D rerio*) in the Light & Dark Test (0−5 min) (B) of the chalcone PAAPMNBA.Native-untreated animals.FLZ-Fluoxetine (0.0.5 mg/kg; 20 µL; *i,p.*). Vehicle(DMSO 3%) (20 µL; *i,p*).Values represent the mean ± standard error of the mean for 6 animals/group; AVOVA followed by *Turkey* (***p < 0,0001; *vs*.Naive; p < 0,05; ##p < 0,01; ###p < 0,001; ####p < 0,0001 *vs*.Vehicle).

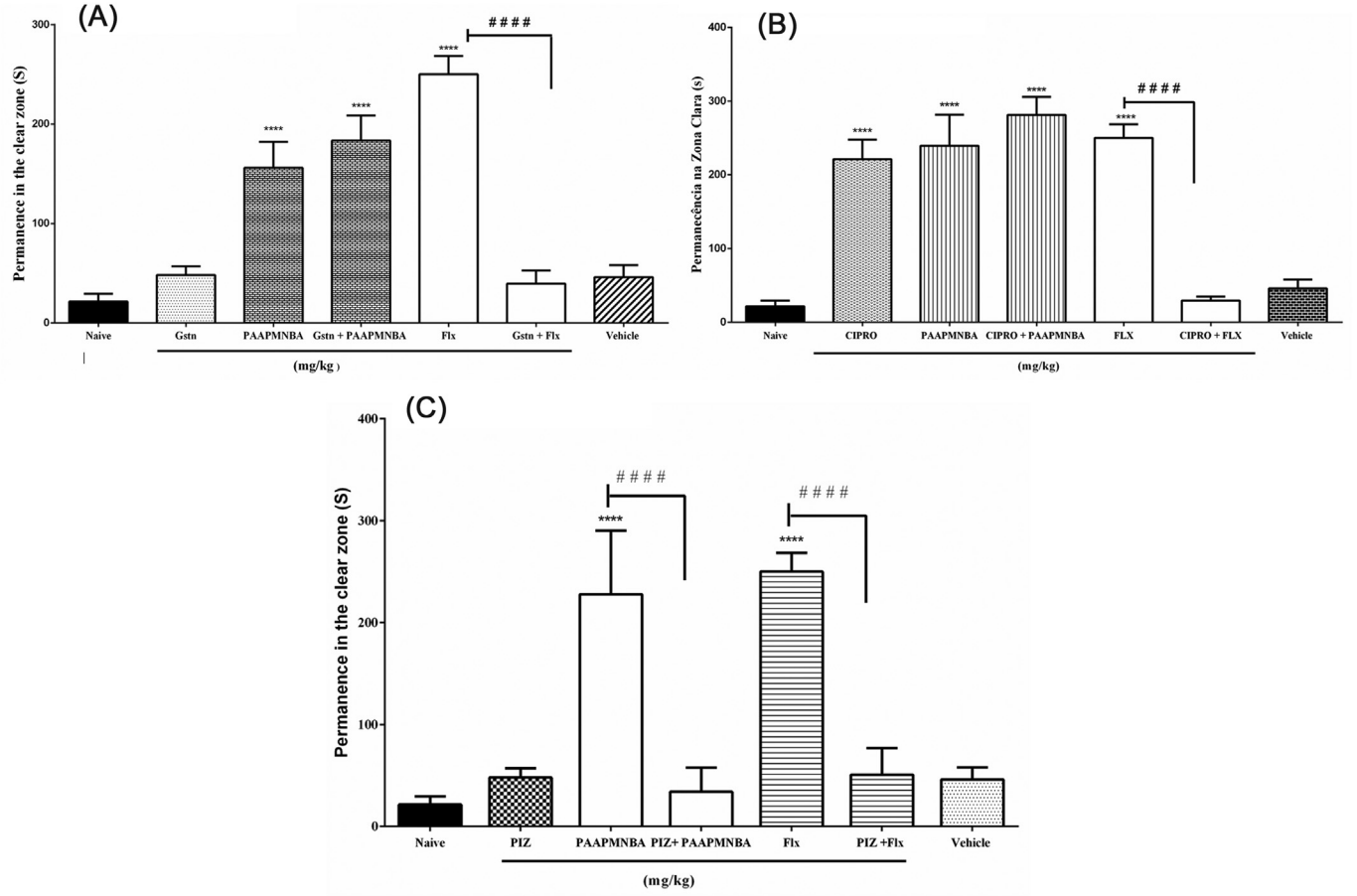

Fig. 3. Effect of granisetron (A), cyp roheptadine (B), and pizotifene (C) under the anxiolytic effect of the chalcone PAAPMNBA in the Light & Dark Test. The results are expressed as mean values ± S E M.(n = 6/group).AVOVA followed by the Turkey test ****p < 0.0001 vs.Ship or Vehicle;####p < 0.0001 vs. PAAPMNBA or Flx).Cipro-cyproheptadine; Grain-granisetron; Piz-pizotifene; Flx-fluoxetine.

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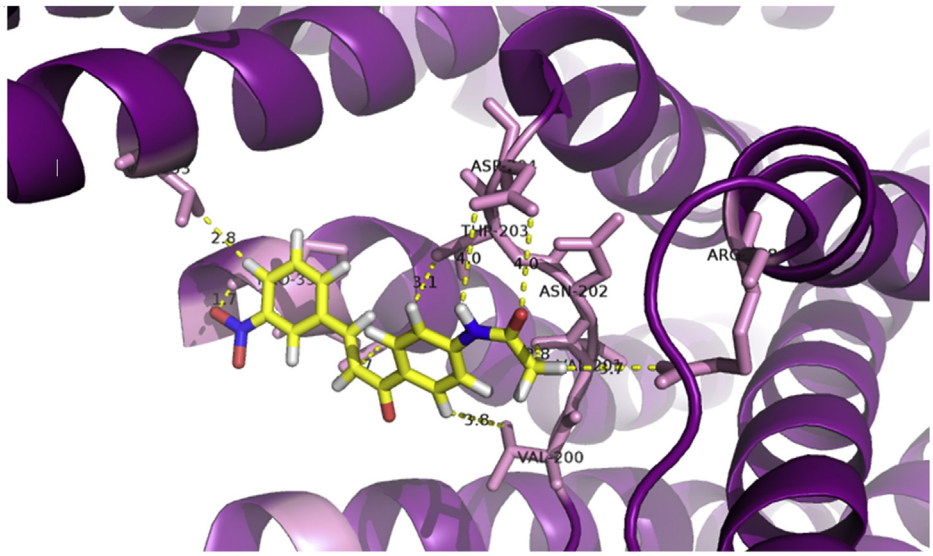

Fig. 4. Amino acid residues involved in the recognition of Chalcone PAAPMNBA by the 5-HT1 receptor: Thr203, Asp204, An202, Arg188, Val201,Val233, Pro338 AN Met337, showing 9 chemical bonds(1.7–4.0 Å) and high conformational stability.

molecule. These data justified the fact that PAAPMNBA contains the C═O conjugated double bond, and that the acetyl group was attached to the $-NH_2$ group of C-4'.

The absorption bands at 1610 and 1660 cm^{-1} in the FT-IR spectrum justified the presence of the carbonyl group of the enona system and the N–C═O group, respectively. The absorption band at 3330 cm^{-1} indicated the presence of an $-NH_2$ group, where the hydrogen atom had been replaced by an acetyl group. High resolution mass data with *m/z*: 310.1445 ($C_{19}H_{19}NO_3$) $[M + H]^+$ (calcd. 310.1443) confirmed the data obtained from the NMR and FT-IR spectra.

Chalcones are the main precursors in the biosynthesis of flavonoids and isoflavonoids and possess numerous biological activities, including antiacetylcholinesterase [20], antioxidant [21], and anxiolytic [22] properties. It was observed that PAAPMNBA was not toxic to zebrafish over a duration of 96 h and decreased their locomotor activity. Similar results were reported for the chalcone, PAAPFBA [8]. Other studies also assessed the toxicity of chalcones in zebrafish, however, the authors had performed the studies using zebrafishes in the embryonic phase [23].

Zebrafish is a model animal that is used for neurobehavioral studies. It is considered to be an excellent model organism for pharmacological studies and/or safety research at an early stage of drug development [24,25]. The open field test was used to evaluate the effect of PAAPMNBA on the locomotor system of adult zebrafish. Analysis of locomotor activity by open field tests can generate a model that can be used to assess hyperactivity, which is an indicator of anxiety [26]. It was observed that PAAPMNBA caused locomotor impairment in the open field test, at all doses. PAAPMNBA induced maximum reduction in locomotor activity at a dose of 40 mg/kg ($P < 0.0001$ vs naive) and decreased the mobility of the animals at doses of 4 and 12 mg/kg ($P < 0.001$ vs. naive). The treatment of zebrafish with anxiolytic drugs can increase their exploratory activity in the open field [27], induce a sedative effect, and decrease their locomotor activity [24,28]. Therefore, the anxiolytic activity of PAAPMNBA was evaluated by the light and dark test.

The light and dark test is generally applied to assess the anxiolytic effect of various substances on rodents. It has been recently adapted and validated for use in zebrafish models. Anxiety-like behavior is induced in zebrafish and rodents in response to similar environmental stimuli. Both animals prefer dark environments to light ones [25,29]. The intraperitoneal (*ip*) administration of PAAPMNBA, at doses of 4, 12, or 40 mg/kg, and fluoxetine, at a dose of 0.05 mg/kg, increased ($P < 0.0001$ vs. vehicle) the time spent by the zebrafish in the clear zone of the aquarium, suggesting an anxiolytic effect (Fig. 2B). The serotonergic agonist, buspirone (a), and ethanol, are also anxiolytic, and have been demonstrated to increase the time spent by the experimental animals in the clear zone [16,30].

The chalcone derivative, 5′-methyl-2′-hydroxichalcona, has also been recently demonstrated to have an anxiolytic effect in mice, as evaluated by the elevated plus-maze assay. This class of compounds holds promise for the development of novel anxiolytic agents, owing to their neuroprotective properties, that be applied for the treatment of pathological conditions, including anxiety and stress [7,31]. Generally, the majority of these anxiolytic compounds carry hydroxyl, methoxy, methyl, dimethylamine, halogens, and nitro substituents [7]. The chalcone, PAAPMNBA, also possesses the nitro and methyl groups (Fig. 1A).

In the present study, the interaction between PAAPMNBA and the 5-HT receptors (5-HTRs), that are involved in anxiety, was investigated. The anxiolytic potential of PAAPMNBA was assessed following pretreatment with cyproheptadine antagonists (a 5-HTR 2A antagonist), pizotifen (a 5-HTR 1 and 5-HTR 2A/2C antagonist), and granisetron (a 5-HTR 3 antagonist). Fluoxetine was used as the positive control. Unlike granisetron (5-HTR 3A/3B; Fig. 3A) and cyproeptadine (5-HTR 2A; Fig. 3B), pretreatment with pizotifen significantly inhibited ($^{\#\#\#\#}p < 0.0001$) the anxiolytic effect of PAAPMNBA, suggesting that the mechanism of action of PAAPMNBA involves the 5-HTR 1 and/or 5-HTR 2A/2C serotonergic receptors (Fig. 3C). It was additionally observed that all the aforementioned antagonists reversed the effects of fluoxetine.

Serotonergic neurotransmission in zebrafish is important for several behavioral responses, including locomotion, motor function [11,32], and responses similar to anxiety [33]. The pharmacological activation of serotonin receptors has been shown to reduce behavioral anxiety measures [34,35]. It has been demonstrated that pretreatment with fluoxetine inhibits anxiogenic effects in the light and dark test, and increases 5-HT in the extracellular environment

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of the cerebrum [36]. Few studies have investigated the effects of different serotonin receptor antagonists on the motor function and behavioral measures similar to anxiety in adult zebrafish [9]. Among the seven major classes of 5-HT receptors, the 5-HTR 1, 5-HTR 2, and 5-HTR 3 receptors are the ones that are most directly involved in anxiety. Drugs that act on the 5-HTR 1A receptor has been shown to decrease anxiety in zebrafish and rodents [36,37].

Molecular docking (Fig. 4) revealed 10 energy clusters for the association between PAAPMNBA and the 5-HTR 1 receptor, which proved the affinity and specificity of PAAPMNBA for the interaction site of 5-HTR 1. It was observed that PAAPMNBA altered the folding of the 5-HTR 1 receptor. This was attributed to the fact that a large number of amino acid residues were involved in recognizing PAAPMNBA, suggesting high ligand stability at the site.

5. Conclusion

The results of the present study confirmed the structure of the PAAPMNBA chalcone through characterization data. This study also provides evidence that the evaluated chalcone is non-toxic to adult zebrafish and has anxiolytic action mediated by the 5-HTR receptor. The interaction of chalcone with this receptor is confirmed by the study of molecular docking.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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