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Pharmacological screening of a new alpha-2 adrenergic receptor agonist, mafedine, in zebrafish

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ABSTRACT

Pharmacological agents acting at alpha-2 adrenergic receptors are widely used in physiology and neuroscience research. Mounting evidence of their potential utility in clinical and experimental psychopharmacology, necessitates new models and novel model organisms for their screening. Here, we characterize behavioral effects of mafedine (6-oxo-1-phenyl-2- (phenylamino)-1,6-dihydropyrimidine-4-sodium olate), a novel drug with alpha-2 adrenergic receptor agonistic effects, in adult zebrafish (*Danio rerio*) in the novel tank test of anxiety and activity. Following an acute 20-min exposure, mafedine at 60 mg/L produced a mild psychostimulant action with some anxiogenic-like effects. Repeated acute 20-min/day administration of mafedine for 7 consecutive days at 1, 5 and 10 mg/L had a similar action on fish behavior as an acute exposure to 60 mg/L. Since mafedine demonstrated robust behavioral effects in zebrafish – a sensitive vertebrate aquatic model, it is likely that it may modulate rodent and human behavior as well. Thus, further studies are needed to explore this possibility in detail, and whether it may foster clinical application of mafedine and related alpha-2 adrenergic agents.

1. Introduction

Alpha-2 adrenoreceptors are G protein-coupled receptors regulating multiple brain functions [1,2]. Activated presynaptic alpha-2 adrenergic receptors suppress the release of neurotransmitters (e.g., noradrenaline, acetylcholine, serotonin and dopamine) from synaptic terminals [3], whereas reduced alpha-2 signaling facilitates their release [4]. Currently, several different subtypes of mammalian alpha-2 receptors have been identified [5], including alpha-2_A [6,7], alpha-2_B [8] and alpha-2_C [9] receptors. Activation of alpha-2_A receptors in mice causes region-specific effects, including hypotension (rostral ventrolateral medulla) [6] or sedation (locus coeruleus) [5]. Alpha-2_A receptors also mediate antinociceptive and hypothermic effects of some alpha-2 agonists, such as dexmedetomidine [1]. Much less is known about physiological functions of the alpha-2_B and alpha-2_C receptor subtypes. For example, while alpha-2_B receptors are involved in the regulation of vascular tone [8], the alpha-2_C subtype does not play a major role in cardiovascular or other classical alpha-2 adrenergic effects, such as dexmedetomidine sedation [5], but may be related to mouse stress-induced depression-like state [10] and cognitive performance [11].

In addition to adrenaline itself, alpha-2 receptors can also bind

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clonidine, dexmedetomidine, tizanidine and other agents [12]. Clonidine is a classical non-selective agonist of alpha-2 adrenergic receptors, acting on all three receptor subtypes [13]. The well-known properties of clonidine and related drugs are antihypertensive, analgesic, sedative and anxiolytic [14]. Alpha-2 adrenergic agonists are also used clinically, to treat several other conditions, including withdrawal syndrome [15], attention deficit hyperactivity disorder (ADHD) [16] and child insomnia [17]. More selective agents from this group (e.g., dexmedetomidine) are also used for premedication in anesthetized patients before surgery (i.e., to reduce their anxiety, sympathoadrenal responses and opioid dosage needed for analgesia [18,19]), and appear to be more effective than clonidine [20]. Alpha-2 agonists, most likely due to their general calming effects, have also been proposed as a preventive measure for delirium that may follow general anesthesia [21].

The developing utility of alpha-2 ligands in biomedicine necessitates novel approaches and in vivo screening tools to further characterize their CNS effects. The zebrafish (*Danio rerio*) is a highly-sensitive biological model system that rapidly gains popularity in biomedicine [22,23]. Because of their low cost and the ability to rapidly reproduce and mature, zebrafish are also emerging as an excellent object for in-vivo neuropharmacological and neurotoxicilogical studies [22,24,25]. In addition, zebrafish possess a fully characterized genome [26,27] and high genetic, anatomical and physiological homology with humans and rodents [26,28]. For example, nearly 70% of zebrafish genes have at least one human ortholog, while nearly 50% of human genes are the same as in this fish [26]. Despite some differences in the CNS anatomy between human and zebrafish [29], they have many structures of the brain that perform similar functions to those in mammals [30]. There is also a high degree of similarity of pharmacological targets and main neurotransmitter systems, including monoaminergic transmission and adrenergic receptors [31–33]

The novel compound, mafedine, is a pyrimidine derivative 6-oxo-1-phenyl-2- (phenylamino)-1,6-dihydropyrimidine-4-olate sodium (Fig. 1) synthesized in the Organic Chemistry Department of St. Petersburg State Chemical Pharmaceutical Academy in St. Petersburg, Russia [34,35]. The drug is a white crystalline powder with pink tinge, highly soluble in water. In a series of studies of its antihypertensive effects, mafedine activity was mediated via central alpha-2 adrenergic receptors [34,35]. Therefore, similar to clonidine and other alpha-2 agonists, mafedine may be effective in certain neurological and mental conditions. Capitalizing on the zebrafish model as a sensitive in vivo system for CNS drug discovery, here we examine the effects of acute administration of mafedine on adult zebrafish behaviors.

2. Materials and methods

2.1. Animals

The study was performed in adult (5–7 months) wild-type short-fin zebrafish obtained from a commercial vendor (Tropical Aquarium, St. Petersburg, Russia), with the male:female ratio in their stock outbred zebrafish population of approximately 50:50. Animal housing adhered to the existing accepted standards of zebrafish care. Animals were kept in groups of 15 fish per a 20-L tank with water filtration system, water temperature set at −25 °C, light at 950–960 lx, with a 10/14-hours cycle of day/night [36]. All animals in this study were experimentally naïve and fed twice a day with Tetramin-Pro (Terta GMBH, Osnabruck, Germany). Behavioral testing was performed between 12.00 and 19.00 h using the novel tank test, representing a plexiglass container (20 × 20 × 5 cm) virtually divided by a marker line into the upper and lower halves [37]. Before the testing, the fish were acclimated for 20 min in small 1-L opaque plastic containers in 0.5 L water or a mafedine water solution at several concentrations. The most commonly used behavioral paradigm in adult zebrafish, the novel tank test is conceptually similar to the rodent open field test and exploits the natural behavior of zebrafish to seek protection in an unfamiliar environment by diving to the bottom and remaining there until they feel safe enough to explore [37]. Using this model helps collect and compare multiple behavioral parameters (e.g., time spent or frequency in top/bottom zones, the number of freezing bouts, velocity and distance traveled) to assess anxiety and activity levels in adult zebrafish. Given putative central/behavior effects of mafedine, the novel tank test was chosen here as a sensitive and reliable phenotyping tool to assess drug-evoked responses in zebrafish.

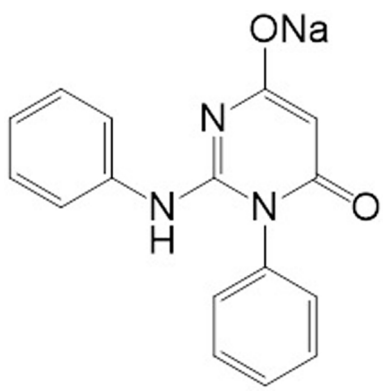

Fig. 1. Chemical structure of mafedine.

2.2. Treatment and behavioral testing

Behavioral testing was performed between 12.00 and 19.00 h. Animals were randomly divided on several experimental groups (n = 15 fish in each group). Experiment 1 tested dose of 60 mg/L of mafedine, comparing it with control fish. Mafedine for this study was obtained from Organic Chemistry Department of St. Petersburg State Chemical Pharmaceutical Academy (St. Petersburg, Russia). A dose of mafedine and a 20-min acute treatment time for Experiment 1 were chosen based on our pilot experiments with this drug and other substances tested in this aquatic model. In Experiment 2 we evaluated the effects of repeated acute exposures to mafedine for 7 consecutive days at 1, 5 and 10 mg/L for 20 min daily. These doses were selected for repeated treatment based on our pilot experiments and also derived from a higher, behaviorally active acute dose (60 mg/L) established previously in Experiment 1.

Trials were recorded for 5 min by a side-positioned web-camera for further offline analyses, and then processed using the Ethovision XT 11.5 software (Noldus Information Technology, Wageningen, Netherlands). For each animal, the distance travelled (cm), the mean and maximum velocity (cm/s) and turn angle (deg) were estimated. Also the frequency and duration (s) of low mobility (the complete area detected as animal is changing with < 20% threshold), high mobility (the complete area detected as animal is changing with > 60% threshold) and not moving state (no changes of location, threshold was defined as: start velocity – 2.00 cm/s, stop velocity – 1.75 cm/s), the time spent in the top and in the bottom zones (s), the frequency of transition from bottom to top and latency time of the first bottom-top transition (s), as described earlier [24].

2.3. Statistical analyses

The data are presented as mean (M) ± standard error mean (SEM). In Experiment 1, data were analyzed using the Mann-Whitney *U* test Statistical significance in multu-group Experiment 2 was assessed using the Kruskal-Wallis test followed by Dunn´s post-hoc test for significant data. The level of confidence was set as 95% for all tests ($p < 0.05$).

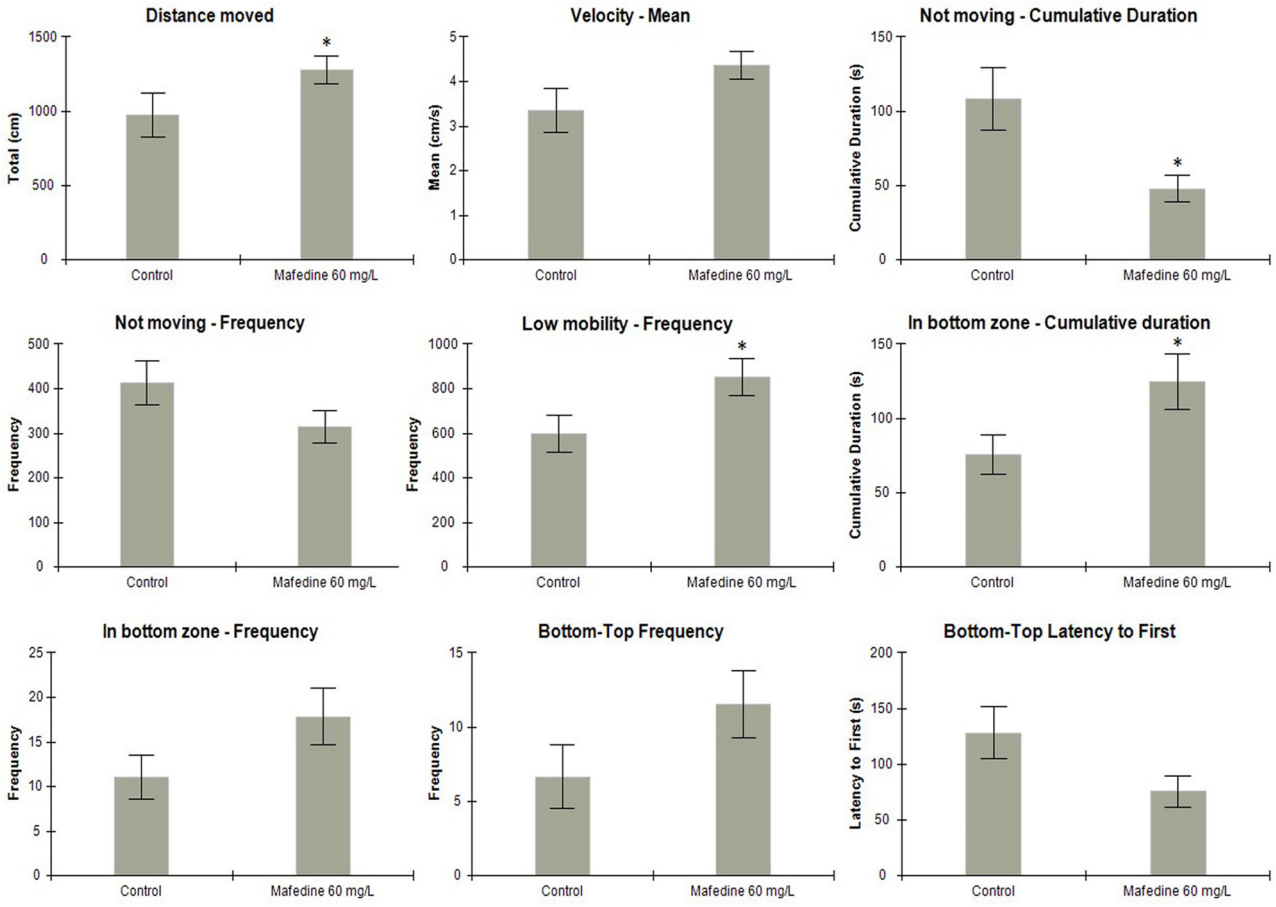

Fig. 2. Behavioral effects of acute 20-min exposure to 60 mg/L mafedine on zebrafish anxiety and activity tested in the novel tank test (Experiment 1). * $p < 0.05$ vs. control, Mann-Whitney *U* test (n = 15 per group).

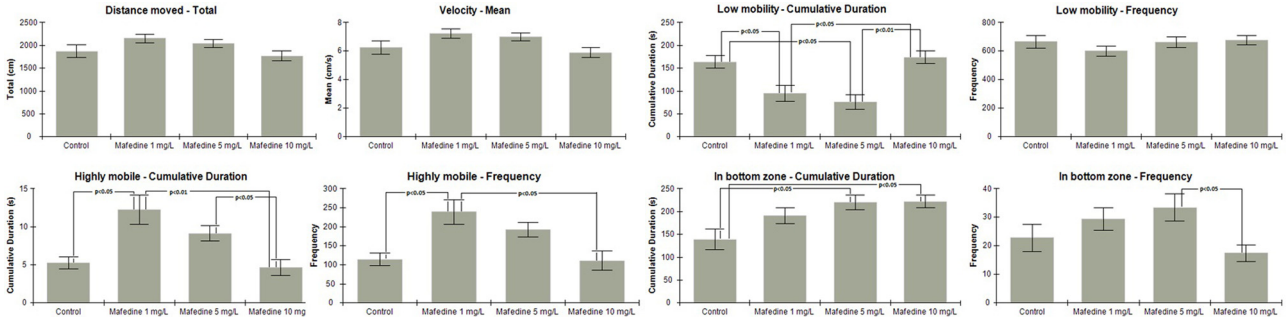

Fig. 3. Behavioral effects of repeated acute 20 min/day exposure to mafedine for 7 consecutive days at 1, 5 and 10 mg/L on zebrafish anxiety and activity tested in the novel tank test (Experiment 2). $p < 0.05$, $p < 0.01$ by Dunn's post-hoc test for significant Kruskal-Wallis data (n = 15 per group).

3. Results

Overall, acute mafedine exposure at 60 mg/L (Experiment 1) showed significantly more traveled distance (U = 64, df = 28, p = 0.045), time spent in bottom (U = 62.5, df = 28, p = 0.037), low-mobility episodes (U = 62.5, df = 28, p = 0.037) but shorter 'not moving' duration (U = 64, df = 28, p = 0.031) (Fig. 2).

In Experiment 2, repeated acute daily exposure to mafedine for 7 days at 1 mg/L reduced low mobility state duration (H = 18.711, df = 3, p = 0.045) and increased high mobility state frequency (H = 14.203, df = 3, p = 0.049) and duration (H = 17.473, df = 3, p = 0.026). The observed mafedine effects were dose-dependent and decreased with higher doses for high mobility state frequency (H = 14.200, df = 3, p = 0.0133, 1 mg/L vs. 10 mg/L) and duration (H = 17.470, df = 3, p = 0.0034 for 10 mg/L vs. 1 mg/L, and p = 0190 for 5 mg/L vs. 10 mg/L). Also mafedine at 5 and 10 mg/L increased time spent in the bottom zone (H = 9.916, df = 3, p = 0.0363 and p = 0.0399, respectively). The bottom zone visit frequency was lower in 10 mg/L group in comparison with 5 mg/L (H = 9.651, df = 3, p = 0.0361) (Fig. 3).

4. Discussion

As pharmacology of alpha-2 adrenergic agonist and antagonist action remains poorly understood [38], their further clinical and preclinical testing *in vivo* becomes important. For example, guanfacine (predominantly, an alpha-2_A agonist in humans [12]) is an anxiolytic treatment for clinical generalized and separation anxiety disorders

Table 1
CNS localization, known physiological functions and relative genetic homology of different alpha-2 adrenergic subtypes in zebrafish and rodents (NA – data not available).

Receptor subtypes							
2A		2B		2C		2 Da/2Db	
Localization	Function	Localization	Function	Localization	Function	Localization	Function
Zebrafish							
Ventral telencephalon, preoptic, pretectal and hypothalamic areas, oculomotor nucleus, locus coeruleus, medial raphe, reticular formation, Purkinje layer of the cerebellum [43]	NA	Hypothalamus [33]	NA	Diencephalon (torus lateralis and optic tectum) [33]	NA	Cerebellum, optic tectum (2 Da) [33]	NA
% homology with mice[a]							
Gene	81%		82%		85%	NA	NA
Protein	59%		52%		66%	NA	NA
% homology with humans[a]							
Gene	77%		78%		[b]	NA	NA
Protein	58%		68%		61%	NA	NA
Rodents (mice, rats)							
Medulla [6], locus coeruleus [5], amygdala, hypothalamus, olfactory system [44], both horns of the spinal cord [45]	Hypotension [6], nociception, hypothermia [1] and sedation [5]	Thalamus, Purkinje layer of the cerebellum [44]	Regulation of vascular tone [8]	Striatum [44], locus coeruleus [46], ventral horns of the spinal cord [45]	Stress behavior [10], memory and spatial navigation [11]	NA	NA
% homology with humans[a]							
Gene	83%		84%		87%	NA	NA
Protein	92%		83%		92%	NA	NA

[a] Genetic (nucleotide sequence) and protein (amino-acid sequence) homology between species was assessed by nucleotide and amino acid sequences using the BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi for *Danio rerio, Mus musculus* and *Homo sapiens*, assessed in October 2018).

[b] No significant homology (% is unavailable), as per BLAST search results generated.

[39]. Another alpha-2 agonist, tizanidine (whose specific selectivity data is yet unavailable in public databases) shows efficacy in spasticity therapy in patients following a traumatic brain injury [40]. Likewise, clonidine (predominantly, an $\alpha 2_A$ agonist in humans [12]) reduces sleep deficits and hyperexcitability in patients with post-traumatic stress disorder (PTSD) [41].

Recently established antihypertensive effects of mafedine have been linked to its putative action at central alpha-2 adrenergic receptors [34,35]. In line with this, an alpha-2 antagonist yohimbine (with mild alpha-1 antagonistic action) prevents mafedine-induced antihypertensive action, whereas selective alpha-1 antagonist prazosin does not [35]. Notably, transection along the caudal border of medulla blocks hypotensive effects of mafedine in rats, indicating its central mechanisms of action [35]. Compared to clonidine, mafedine has slower and more prolonged hypotensive effects, and its cessation does not lead to 'clonidine withdrawal'-like hypertension [42]. Paralleling clinical findings, previous mouse studies show that mafedine at 100 mg/kg causes sedation and hypolocomotion, whereas its higher doses (> 1000 mg/kg) evoke sleep [35]. Moreover, 100 mg/kg mafedine accelerates the onset and increases duration of mouse sleep induced by a hypnotic agent barbamil [35]. Thus, similar to clonidine and other alpha-2 agonists, it is possible that mafedine may also exert clinically relevant CNS effects in some neurological and mental conditions that merit further scrutiny and preclinical testing.

Indeed, pilot studies suggest anxiolytic and hypnotic effects of a putative novel alpha-2 agonist mafedine in mice [35]. The present study is the first report of behavioral effects of mafedine in zebrafish. To the best of our knowledge, other alpha-2 agonists have not previously been tested in zebrafish. Notably, unlike rodents, zebrafish express five subtypes of alpha-2 receptors (2_A, 2_B, 2_C, 2_{Da} and 2_{Db}) [32] that exert different physiological effects but remain largely unexplored (Table 1). While earlier rodent data [34,35] and the present zebrafish results (Figs. 2 and 3) suggest neurotropic activity of mafedine, in humans this drug was studied only as an antihypertensive agent (in 100-mg tablets), even though during the clinical trial some patients did note sedation and drowsiness [42].

Since the novel tank test examines both general locomotion and anxiety levels in adult zebrafish [22], the use of this model here was well justified, conceptually paralleling the established novelty-based rodent models [28]. In general, longer distance traveled and higher mean velocity indicate a moderate psychostimulant action of 60 mg/L mafedine in zebrafish (Fig. 2), which is not common for other alpha-2 agonists tested in mice [47–49]. Indeed, acute administration of 50 μg/kg clonidine reduces mouse locomotion [47] with a similar action reported 24 h following administration of 2 mg/kg of guanfacine [48]. Likewise, a highly-selective alpha-2 agonist dexmedetomidine at 5, 10 and 20 mg/kg, also reduces mouse motor activity 20 min after the injection [49].

Since zebrafish treated with 60 mg/L stayed longer at the bottom of the tank (Fig. 2), a possible explanation for this response can be a mild anxiogenic-like action of mafedine. This possibility is supported by the fact that low mobility frequency was also significantly higher vs. controls, and that these effects cannot be associated with non-specific sedation/hypoactivity in fish, because mafedine exerts a moderate psychostimulant action in parallel. Interestingly, however, the observed putative anxiogenic-like effects if mafedine are consistent with its psychostimulant action, since the psychostimulants usually increase anxiety in both experimental animals [50–52] and humans [53,54].

Furthermore, although anxiogenic action is not a hallmark behavioral effect of alpha-2 agonists in mammals, several studies using different stress models in rodents demonstrate complex dose-dependent anxiotropic effects of another alpha-2 agonist, clonidine. For example, it is anxiolytic in the Vogel conflict test and the elevated plus maze at low doses (6.25–10.0 μg/kg), but causes anxiogenic effects at higher doses (12.5–80.0 μg/kg) [55]. Furthermore, a selective alpha-2 adrenergic antagonist idaxozan blocks anxiolytic effects of clonidine, but not its anxiogenic effects, whereas co-administration of prazosin (an alpha-1 receptor antagonist) with clonidine blocks its anxiogenic, but not anxiolytic, action [55]. In the mouse sleep deprivation model, clonidine shows similar profile in the elevated plus maze, being anxiolytic at low doses (5 and 10 μg/kg) and anxiogenic at higher (50 and 100 μg/kg) doses [56]. A similar biphasic action of clonidine is also seen in control animals, as 5 and 10 μg/kg are ineffective, 50 μg/kg anxiolytic, and 100 μg/kg – anxiogenic [56].

Moreover, since additional action at alpha-1 receptors (e.g., already shown for clonidine [57,58]) may play a role in such complex behavioral effects, similar action may be expected from mafedine. For instance, paralleling anxiogenic effect of clonidine (high doses likely mediated via apha-1 receptors [55]), it is possible that mafedine at 60 mg/kg exerts similar effects. This is indirectly supported by the fact that lower doses of mafedine in our pilot preliminary studies did not alter zebrafish anxiety or activity (data not shown). However, the alpha-1 adrenergic receptors may not be the main target for mafedine and other alpha-2 adrenergic agonists. For example, an alpha-1 adrenergic agonist phenylephrine injection into rat nuclei accumbens shows no behavioral effects in the elevated plus maze [59]. In addition, as already mentioned, neither agonists nor antagonists of alpha-1 adrenergic receptors are effective in the treatment of clinical anxiety disorders [60].

Overall, the present findings suggest that mafedine exerts a moderate psychostimulant action in zebrafish, as evidenced by their longer distance traveled and mild acceleration in the novel tank tast. However, the drug also increases time spent in bottom (in both acute and repeated experiments) and frequency of low mobility state episodes (in Experiment 1), which suggests a likely anxiogenic-like profile. Since the drug demonstrated robust behavioral effects in zebrafish, it is likely that it may affect rodent and human behavior as well. Thus, further studies are needed to explore this possibility in different model organisms, especially rodents, better characterize and interpret the observed mafedine effects. Finally, the putative mild stimulant action of mafedine raises additional possibility of its clinical applications. For example, such moderate psychostimulant action can be beneficial for correcting asthenia which often occurs in various CNS pathologies, such as traumatic brain injury [61] and stroke [62]. Direct action on alpha-2 adrenergic receptors subtypes can lead to benefits of patients' functional outcome without adverse sedative side effects. Collectively, this calls for further research into the exact role of each receptor subtype in the regulation of CNS fuctions and the selectivity spectra of mafedine and related alpha-2 adrenergic drugs.

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