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Effects of novel hexahydropyrimidine derivatives as potential ligands of M1 muscarinic acetylcholine receptor on cognitive function, hypoxia-induced lethality, and oxidative stress in rodents

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ABSTRACT

The neurodegenerative diseases have a complex pathogenetic mechanism comprising oxidative stress and receptor system dysfunction caused by various damaging factors such as, for example, brain hypoxia. The purpose of this study was to elucidate the influence of hexahydropyrimidine derivatives on learning, memory, and orientation and locomotor activities in the passive avoidance (PA) and open field (OF) tests and to evaluate these compounds for their potential antihypoxic and antioxidant action on normobaric hypercapnic hypoxia and toxic hypoxia models. We demonstrated that compounds **1a** and **1e** administered as a single 100 mg/kg dose (p.o.) one hour before the tests increased the latency time to enter the dark compartment for the first time and reduced the time spent in the dark compartment on the 2nd, 7th, and 14th days of PAT and increased the number of squares crossed and hole-pokings in the OF test. It was also shown that single administration of compounds **1a** and **1e** (in 100 mg/kg dose, p.o.) one hour before generation of hypoxia increased the life span of mice under normobaric hypoxia by 30% ($P < 0.05$) and, after injection of sodium nitroprusside, they decreased the malondialdehyde (MDA) level and increased the catalase level in the brain of mice. According to molecular docking results, compounds **1a** and **1e** are bound in the orthosteric active site of M1 muscarinic receptor *via* supramolecular interactions with a number of functional amino acids.

The results indicate that hexahydropyrimidine derivatives have a beneficial effect on the memory, learning processes, and orientation and locomotor activities of rats in an unfamiliar environment and exhibit antihypoxic and antioxidant activities under hypoxia in mice. The cognitive enhancement can be mediated by the effect of lead compounds on the M1 muscarinic acetylcholine receptor.

1. Introduction

Neurodegenerative diseases (NDDs) cover a large class of disorders caused by gradual death of particular groups of nerve cells and characterized by progressive neurologic disorders, including locomotor impairment, psychoemotional and cognitive (up to dementia) impairment, and epileptic seizures. One of the factors inducing the development of NDDs is hypoxia [1]. Hypoxia may be caused by various circumstances such as ischemic stroke or exposure to toxic agents [2,3]. Hypoxia enhances the formation of reactive oxygen species (ROS) and lipid peroxidation (LPO) and reduces the functional activity of mitochondria and, as a consequence, impairs nerve cell functioning [4].

One more factor considered as inducing NDDs is cholinergic dysfunction caused by deficiency of acetylcholine in the brain cortex and hippocampus, which also causes memory and cognitive impairment [1]. The M1 and M4 subtypes of acetylcholine receptor are of interest as potential biological targets for the treatment of NDDs including Alzeimer's disease, schizophrenia, and narcotic addiction, as they are located in the hippocampus and cerebral cortex, which are the brain areas responsible for learning, memory, and cognitive activity. Unlike the M2 and M3 receptor subtypes, the M1 and M4 subtypes do not affect the functional activity of the cardiac muscle and gastrointestinal tract, which reduces the probability of side effects [5,6].

In view of the complex pathogenetic mechanisms of neurodegenerative diseases (NDDs), in particular, Alzheimer's disease, a topical issue is the search for compounds possessing multifunctional action for

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the NDD therapy [7,8]. Hexahydropyrimidines are the most promising compounds of this type. They were shown to exhibit a broad spectrum of biological activities [9–22]. A number of tetra- and hexapyrimidines were found [23] to be biologically active and exhibit nootropic and anticonvulsant properties. Compounds possessing inhibitory activity towards acetyl- and butyrylcholinesterase and β-secretase-1, which are involved in the pathogenesis of Alzheimer's disease, were found among fluorinated hexahydropyrimidine derivatives [7] (Panek et al., 2017). W.S. Messer, Jr. and co-workers (1997) demonstrated that some tetrahydropyrimidine derivatives are active towards muscarinic acetylcholine receptors, in particular, M1 receptor subtype [1,24].

This study aimed at elucidating the effect of new hexahydropyrimidine derivatives on the cognitive functions and memory using PA and OF tests in rats and on the life span of mice under normobaric hypercapnic hypoxia and hypoxia induced by toxic agents (sodium nitroprusside and sodium nitrite) and at evaluating the antioxidant properties of the identified lead compounds under toxic hypoxia. Since it was shown in similar studies [1,24] that some pyrimidine derivatives can exhibit a nootropic activity by affecting the muscarinic acetylcholine receptors, we estimated the affinity of these compounds to the M1 acetylcholine receptor.

2. Materials and methods

2.1. Starting compounds

The synthesis of hexahydropyrimidine derivatives **1a-e** (Fig. 1) was described previously [25,26]. All compounds were isolated in a pure state and fully characterized, with their physicochemical characteristics being described.

2.2. Animal studies

2.2.1. Animals

The experiments were performed on 162 female Wistar albino rats weighing 170–200 g and 132 female outbred albino mice weighing 18–20 g. The rodents were received from a laboratory animal breeding farm Rappolovo (Leningrad Region, Russia). The animals arriving from the breeder were subjected to a 10-day adaptation period being kept in cages (5 animals per cage) equipped with steel grating covers with a feeding pit and steel separating bar for food. The animals were kept at 21–23 °C, with temperature and humidity being checked every day, and under 12 h light/12 h dark illumination cycle, on a standard diet with free access to food and water. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 2011) and were approved by the Commission for Biomedical Ethics of the Ufa Institute of Chemistry – Subdivision of the Ufa Federal Research Centre of the RAS.

2.2.2. Behavioral study

2.2.2.1. Passive avoidance test. The influence of the test compounds on passive avoidance learning and memory was investigated using an apparatus(Panlab, Spain) consisting of two communicating chambers, one being illuminated and one being darkened with the floor connected to power supply. The procedure includes two phases: habit learning and demonstration of habit retention. On the first day (learning phase), a rat was placed in the middle of the illuminated chamber with its tail towards the hole into the dark chamber. Then the latency to enter the dark chamber (the time elapsed between placing the rat in the middle of the illuminated chamber and the rat entering the dark chamber for the first time) was recorded, with the total time of observation being 5 min (the rats that did not enter the dark space during this period were excluded from the test). After the rat entered the dark chamber, it was subjected to foot shock (0.3 mA, 9 s) with a closed guillotine door. The second phase (retention trial without foot shock) was performed after 24 h and then on the 7th and 14th days after learning [27]. The latency before the first entry, the total time the rat spent in the dark chamber, and the number of rats that did not enter the dark chamber were determined.

2.2.2.2. Open field test. The orientation (exploratory) and locomotor activity of rats were investigated in the *open field* apparatus (OpenSience, Russia), which represented a round arena 95 cm in diameter divided into 16 outer and 8 inner sectors with holes and fenced by a 35 cm high wall. A rat was placed in the middle of the field and its locomotor activity was watched for 3 min by an expert observer. The horizontal activity was measured as the number of crossed sectors,

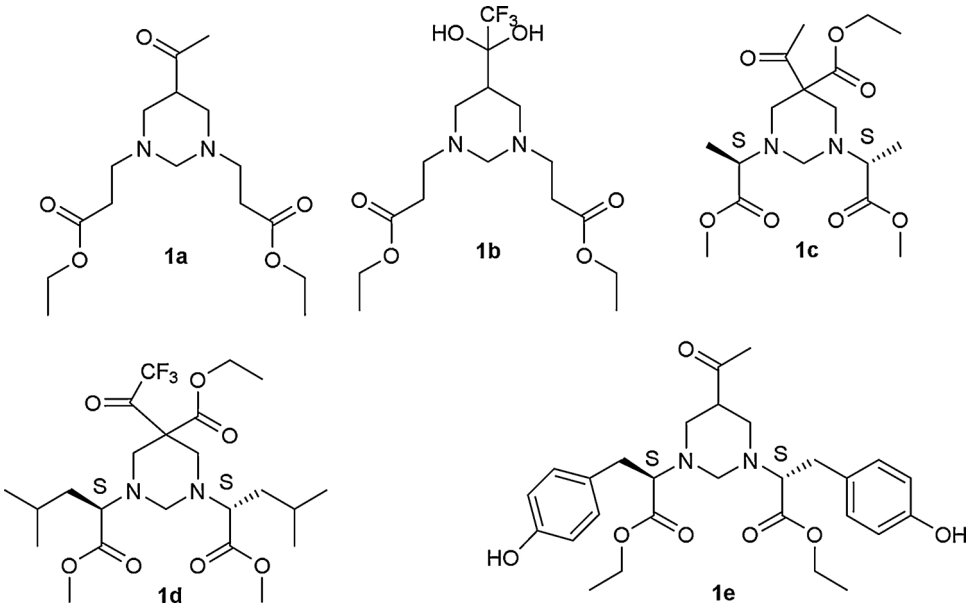

Fig. 1. Hexahydropyrimidines.

while the vertical activity included counting the rearing. The emotional state was determined by anxiety level in the rats, and was assessed by the number of grooming episodes. Spontaneous exploratory behavior was assessed by the number of holes examined [28]. This approach provides an integrated characteristics of the affective state of the rodents whose open-field activity is formed as a resultant of two opposing forces, fear of unfamiliar environment and exploratory motivation [27].

2.2.3. Hypoxia

The normobaric hypercapnic hypoxia was modeled in 200 cm^3 sealed chambers. Mice were placed, one at a time, into a glass box, which was tightly closed and then the oxygen content gradually decreased and pCO_2 in air increased due to breathing of the animal [29]. *Acute histotoxic hypoxia* was induced by the intraperitoneal injection of sodium nitroprusside (25 mg/kg), and *acute hemic hypoxia* was induced by subcutaneous injection of sodium nitrite as a methemoglobin forming agent (240 mg/kg) [30]. The control hypoxic animals were given water in an equivalent volume. The life span of animals was counted off from the time point of hypoxia induction. The life span under hypoxia was recorded in minutes; the activity of compounds was evaluated from the increase in the life span of the test animals with respect to the control. The results were expressed in percent. At the end of the experiment, the mice were sacrificed by decapitation. The brain was processed for further biochemical analysis of oxidative stress markers.

2.2.4. Drugs and treatments

The new hexahydropyrimidine derivatives (**1a-e**) were introduced in the screening dose of 100 mg/kg (for lead compounds **1a** and **1e**, this dose was 1/50 of the maximum dose administered to determine the acute toxicity), with piracetam (CAS 7491-74-9) in 400 mg/kg dose (1/30 of LD50) being used as the reference agent. Piracetam was chosen for this purpose as it has a wide scope of application in the therapy of cognitive impairment [31]. Compounds **1a-d** were dissolved with addition of polysorbate-80, compound **1e** was dissolved with dimethyl sulfoxide (DMSO) with subsequent addition of water. The control animals received the diluting solution (0.1% DMSO solution) in an equivalent volume. The compounds were administered orally 1 h before the tests. Commercial (Sigma Aldrich) chemicals were used.

2.2.5. Preparation of brain homogenates

After the tests, the animals were immediately euthanized *via* cervical dislocation and decapitated and the brain was extracted, weighed, and homogenized in 3 mL of cold tris-HCl buffer (pH 7.4). The biochemical assays were carried out immediately.

2.2.6. Lipid peroxidation measurement

The content of products reacting with thiobarbituric acid (malondialdehyde) in the brain homogenate supernatants was determined using 0.8% 2-thiobarbituric acid. First, 17% trichloroacetic acid (1 mL) was added to the homogenate (2.5 mL). The precipitate was separated by centrifugation (10 min, 4000 *g*, 4 °C). A 0.8% aqueous solution of thiobarbituric acid (1 mL) was added to the supernatant (2 mL). The samples were incubated in a boiling water bath until pink color appeared (the trimethine complex formed) and cooled down to room temperature, and the absorbance at 532 nm was measured [32]. The results were presented as nmol/g of wet tissue [33].

2.2.7. Catalase activity measurement

The antioxidant defense (AOD) level was evaluated from the catalase activity in brain homogenates. The homogenate (0.1 mL) was added to a 0.03% hydrogen peroxide solution (2 mL). In the case of blank sample, distilled water (0.1 mL) was used instead of the homogenate. After 10 min, the reaction was terminated by addition of a 4% ammonium molybdate solution (1 mL). The absorbance was measured at 410 nm against the reference sample (containing 2 mL of distilled water instead of hydrogen peroxide) [34,35]. The catalase activity was expressed as Units (U)/g of wet tissue (1U decomposes 1 mmol H_2O_2/sec) [33].

2.2.8. In vivo acute toxicity

The acute toxicity of hexahydropyrimidine derivatives was determined only for the identified lead compounds **1a** and **1e** upon a single dose intragastric administration to albino female mice. Each dose was tested on five animals in groups. The compounds were administered orally as single doses in solutions containing polysorbate-80 (**1a)** and DMSO (**1e)** in 2500 mg/kg to 5000 mg/kg amounts. The toxic action of the doses was recorded during 24 h. The subsequent observation was continued for 14 days. During this period, the type and duration of the signs of toxicity were noted, including the overall condition, convulsions, and food and water intake [36].

2.2.9. Statistical analysis

Statistical analysis was performed using STATISTICA 10.0 software. The data were expressed as mean ± standard error of mean (SEM). Biochemical data were analyzed using one-way ANOVA with post-hoc Fisher's test. Data behavioral studies were analyzed using Kruskal-Wallis test with post-hoc Mann–Whitney U-test to evaluate differences between individual pairs of experimental groups and Wilcoxon test to evaluate differences within the groups. The differences between groups were considered statistically significant at $p < 0.05$.

2.3. Molecular docking

2.3.1. Active site analysis

The molecular modeling was performed for the orthosteric active binding site corresponding to the PDB code 5CVX [5]. The active site contains functional aromatic amino acid residues such as tryptophan (W157, W378) and tyrosine (Y106, Y404), which were involved π-π and π-cation stacking contacts with the native ligand, and aspartic acid and asparagine in positions 105 and 382 capable of forming hydrogen bonds and salt bridges.

2.3.2. Ligand and receptor preparation

The geometric parameters of the protein complexes with the native ligand were retrieved from the noncommercial Protein Data Bank [37]. Water and other low-molecular-weight molecules within a 5 Å radius around the native ligand were removed and hydrogen atoms were added to amino acid residues. The constrained optimization of the structures was done using the OPLS3 force field [38] at pH = 7.0 ± 0.2.

The hexahydropyrimidine derivatives being considered can be protonated at the nitrogen atom to give β-carbonyl ammonium ions. The protonated molecule is, most often, able to form additional hydrogen bonds and/or salt bridges. The acidity constants for the nitrogen atoms at physiological pH were estimated by the force field method.

The protein and ligand preparation (optimization of all possible conformations and protonated forms) and the docking into the active sites were performed using the Schrödinger Suite 2018-1 software (*Small-Molecule Drug Discovery Suite, 2018-1*).

2.3.3. Docking procedure

Docking was done for flexible conditions (flexible ligand and protein) using the Induced Fit Docking (IFD) protocol with extra prediction accuracy. The size of grid matrix around the native ligand was 15 Å. The ligand-to-protein binding energies were considered as weighted average values taking account of the ratio of protonated and non-protonated forms of hexahydropyrimidine derivatives estimated by the Henderson-Hasselbach equation [39].

The re-docking of the native ligand into the orthosteric active site adequately reproduced the geometric parameters obtained by X-ray crystallography. The RMSDs did not exceed 0.842 (SM).

3. Results

3.1. Behavioral tests

3.1.1. Passive avoidance test

According to the data obtained, on the 2nd day the latent period of the first entry into the dark compartment increased in all groups compared with the day of training. A stastistically significant change from baseline (Wilcoxon test) was found for groups **1a** ($p < 0.0277$), **1c** ($p < 0.0460$), then, in decreasing order of time, in groups **1d** ($p < 0.0277$), piracetam ($p < 0.0130$), and **1e** ($p < 0.0460$). In group **1b**, the latent period was shorter than in the control, but the data were not statistically significant.

According to assessment of the effect of hexahydropyrimidine derivatives on the retention of memory trace on the 7th day, a statistically significant change relative to the control (Mann–Whitney U-test) was found for piracetam ($p < 0.0087$) and compound **1a** ($p < 0.0043$). On the 14th day, piracetam ($p < 0.0152$) and compounds **1e** and **1a** (the data were not statistically significant) increased the latency to enter the dark compartment relative to the control (Fig. 2A).

One more criterion of the memory trace retention in rats, namely, the time spent in the dark chamber on the 2nd day was reduced in the groups **1a**, **1c-1e** and piracetam relative to the control group (the data were not statistically significant). On 7th and 14th days an increase in the time spent in the dark chamber was observed in all groups. This indicator had a shorter time on 7th day in the group of animals that were treated with **la** ($p < 0.0453$) and on 14th day of the experiment in groups **1e** ($p < 0.0082$) and piracetam ($p < 0.0202$) relative to the control (Mann–Whitney U-test) (Fig. 2B).

In the memory trace measurement on the 2nd day of the test, rats that did not visit the dark chamber were observed in all groups. On the 7th, and 14th day of the test but no such rats were in the control group (Fig. 2C).

For the identified lead compounds in the PAT model, **1a** and **1e**, the effective dose (SM) was determined.

3.1.2. Open field test

The open field test was designed to evaluate the effect of hexahydropyrimidine derivatives on the number of squares crossed (**1a** and **1e**) and the number of rearings and pokings into holes for the test groups relative to the control group. The number of grooming events decreased for the test groups. The results were not statistically significant in terms of the Mann–Whitney U-test (Fig. 3A–D).

3.2. Hypoxia models

The effect of hexahydropyrimidine derivatives on the life span of mice under hypoxia was determined. Under experimental normobaric hypercapnic hypoxia, the reference compound, piracetam, and compounds **1c-d** increased the life span of mice by 11%, 13%, and 16%, respectively, relative to the control. The treatment with **1a** and **1e** resulted in the life span of mice under hypoxia being 30% and 34% [$F(142) = 4.697$, $p = 0.0359$] with respect to the control (Fig. 4).

Compounds **1a** and **1e**, which exhibited antihypoxic activity on the model of normobaric hypercapnic hypoxia, were studied on sodium nitroprusside- and sodium nitrite-induced hypoxia models. Sodium nitroprusside uncouples the mitochondrial respiratory chain and, hence, depresses the energy exchange. In our study it enhances lipid peroxidation (LPO) [$F(1,27) = 11.305$, $p = 0.0023$] compared with intact animals. According to the results, hexahydropyrimidine derivatives **1a** and **1e** taken in 100 mg/kg dose increase the life span of mice under histotoxic hypoxia by 16% relative to the control. Compounds **1a** [F

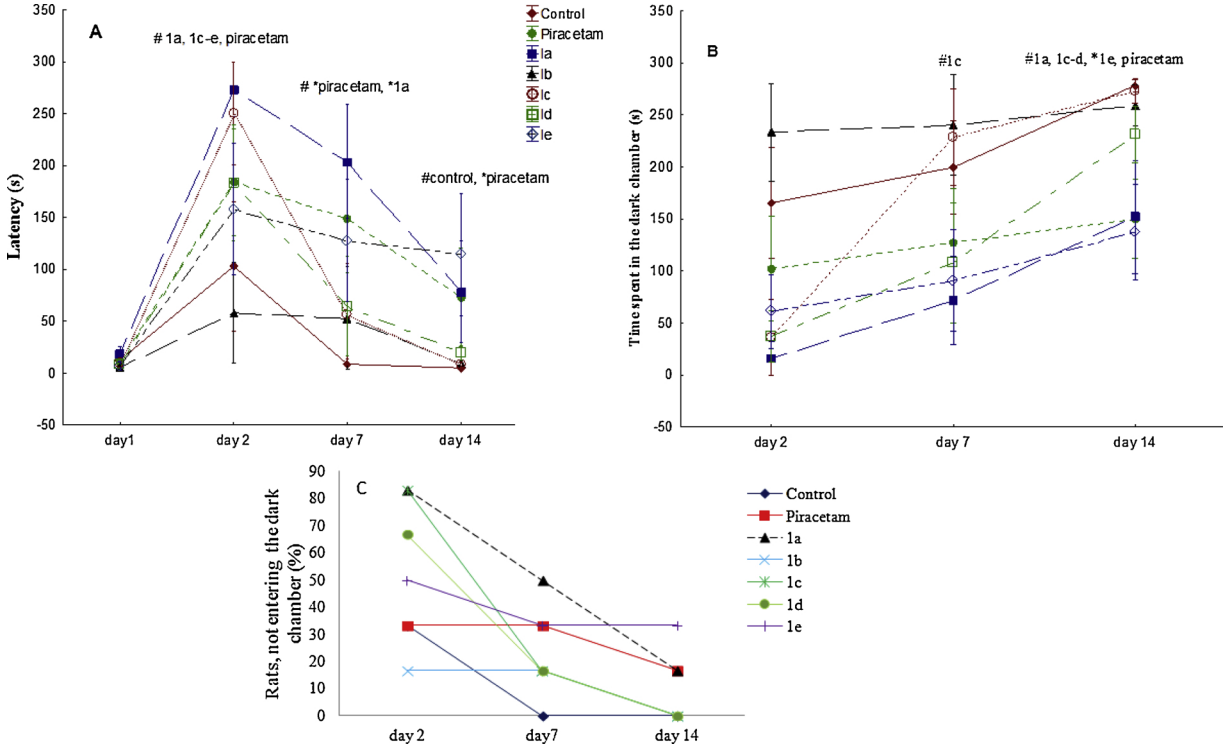

Fig. 2. Effect of hexahydropyrimidine derivatives on the PAT model in rats: (A) latency to enter the dark chamber (mean ± SEM); Day 1: Kruskal-Wallis-test [H (6, N = 42) = 6,570923 p = 0,3624]; day 2: [H (6, N = 42) = 10,61767 p = 0,1009]; day 7: [H (6, N = 42) = 13,41333 p = 0,0369]; day 14: [H (6, N = 42) = 6,822760 p = 0,3376]; (B) time spent in the dark chamber (mean ± SEM);); day 2: Kruskal-Wallis-test day 2: [H (6, N = 42) = 15,49592 p = 0,0167]; Day 7:[H (6, N = 42) = 10,05549 p = 0,1223]; day 14: [H (6, N = 42) = 17,29821 p = 0,0082]; (C) number of rats that did not enter the dark compartment (%). Compounds 1a-e were taken in 100 mg/kg dose, and piracetam dose was 400 mg/kg. Numbers of rats in the groups (n = 6) *The Mann–Whitney U-test was significant at $p < 0.05$ relative to the control. # The Wilcoxon test was significant at $p < 0.05$ relative to the data of the days 1 (A) and 2(B).

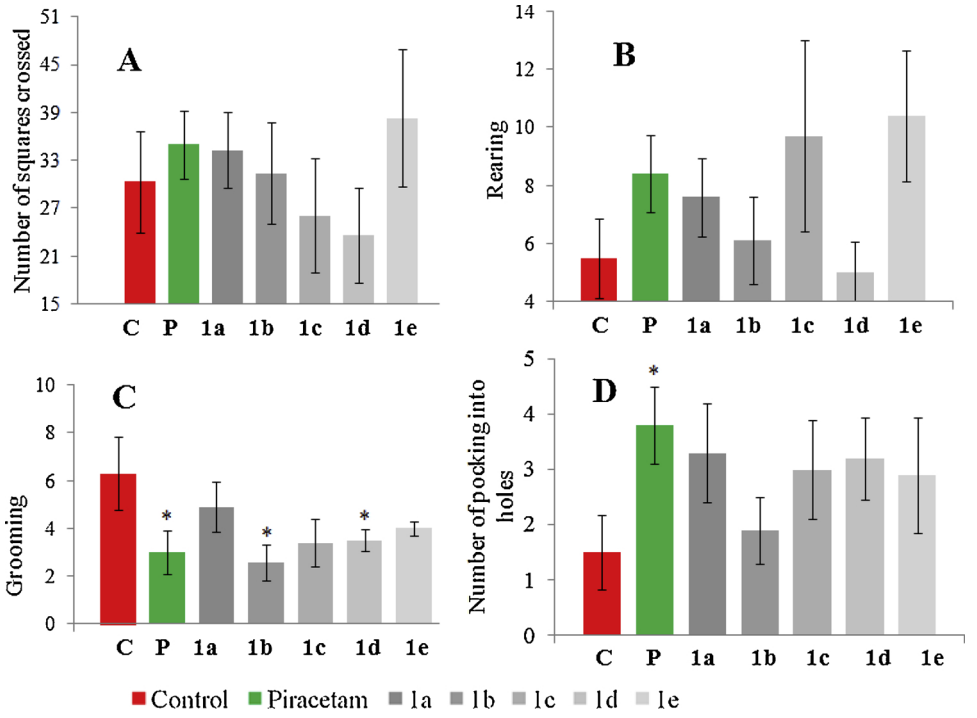

Fig. 3. Effect of the hexahydropyrimidine derivatives in the open field test: (A) horizontal locomotor activity; Kruskal-Wallis-test [H (6, N = 48) = 4.616631, p = 0.5938]; (B) vertical activity: the number of rat rearing events Kruskal-Wallis-test [H (6, N = 48) = 8.595929, p = 0.1976]; (C) the number of grooming events; (D) the number of hole-pokings Kruskal-Wallis-test [H (6, N = 48) = 6.955543, p = 0.3250]. The Mean ± SEM values are presented. Numbers of rats in the groups: 1a-e piracetam (n = 7), control (n = 6). *The Mann–Whitney U-test was significant at $p < 0.05$ relative to the control.

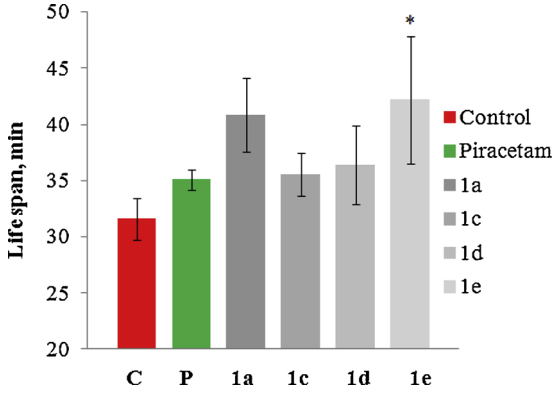

Fig. 4. Life span of mice under normobaric hypercapnic hypoxia upon administration of compounds 1a and 1c-e in 100 mg/kg dose (Mean ± SEM, n = 8). *p < 0.05 (Fisher's test) is statistically significant relative to the control.

Table 1
Effect of hexahydropyrimidine derivatives on the life span and biochemical values under histotoxic and hemic hypoxia in mice.

Compound	Life span, min	MDA level,[a] nmol/g	Catalase level, U/g
histotoxic hypoxia (sodium nitroprusside)			
Intact control	–	10.31 ± 0.93*	8.03 ± 2.20
Hypoxia control	9.71 ± 1.09	28.52 ± 5.7	25.24 ± 2.74
Piracetam	9.26 ± 0.40	8.12 ± 0.90*	10.28 ± 1.19
1a	11.42 ± 0.17	15.24 ± 2.27*	39.94 ± 9.67* ** #
1e	10.91 ± 0.87	14.55 ± 5.41*	30.63 ± 7.55** #
hemic hypoxia (sodium nitrite)			
Hypoxia control	31.01 ± 2.1	17.68 ± 4.33	38.19 ± 5.34
Piracetam	27.4 ± 2.4	8.35 ± 1.82*	12.3 ± 4.25*
1a	23.9 ± 2.40	22.74 ± 5.40	23.41 ± 6.56*
1e	32.28 ± 4.41	19.55 ± 3.0	20.95 ± 4.90*

The presented values correspond to Mean ± SEM (n = 8); *p < 0.05 (Fisher's test) results are statistically significant relative to the hypoxia control, ** - *vs* piracetam, # - *vs* intact control [a)] MDA is malondialdehyde.

(1,27) = 8.076, p = 0.0084] and **1e** [F(1,27) = 7.825, p = 0.0094] and piracetam [F(1,27) = 16.690, p = 0.0004] actively suppress LPO, thus decreasing the level of MDA, a secondary LPO product, in mouse brain homogenates; simultaneously, the catalase level is high in test groups, which attests to AOD activation (Table 1). Thus, treatment with **1a** increased catalase level [F(1,26) = 6.521, p = 0.0169]. In the case of hemic hypoxia induced by sodium nitrite, the test compounds have no effect on the mouse life span. After the action of test compounds, the LPO processes in brain predominate over the AOD. The reference agent piracetam has no effect on the life span of mice under hemic or histotoxic hypoxia, but adjusts the relationship between LPO and AOD to the intact control level (Table 1).

3.3. Acute toxicity

The acute toxicity of the identified lead compounds **1a** and **1e** was evaluated for mice. According to the assay, the compounds in a dose of 2500 mg/kg caused no mortality in mice. In the group receiving **1a** in a 5000 mg/kg dose, dead mice were noted on the fourth day of observation (3 animals out of 5). During the subsequent 10 days, the survived mice developed no symptoms of intoxication. In the group that received **1e** in 5000 mg/kg dose, no mortality was observed during 14 days. The LD_{50} values for the compounds were not determined because of too low toxicity. The maximum oral dose was 5000 mg/kg for both compounds.

3.4. Molecular docking results

The degree of influence of hexahydropyrimidine derivatives on the M1 muscarinic acetylcholine receptor was determined using molecular docking procedure in combination with regression model. As the basic compounds for the regression model, we chose M1 receptor agonists [40] obtained by structural modification of ML071, a highly selective M1 agonist [41].

The selection of these compounds was based on analysis of the

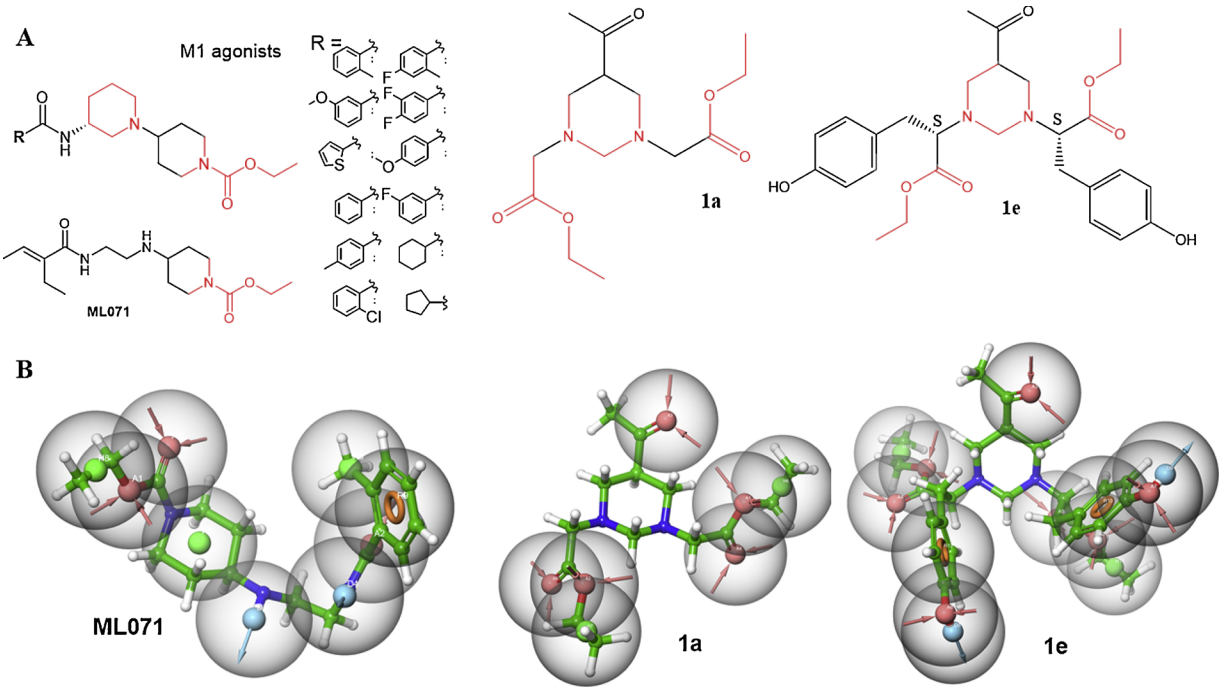

Fig. 5. Comparison of M1 receptor agonists and lead hexahydropyrimidine derivatives (1a and 1e): (A) analysis of structural descriptors; (B) analysis of pharmacophoric profiles: red and blue color show hydrogen bond acceptors and donors, respectively; the hydrophobic parts of the molecules are denoted by green; the aromatic rings are given in orange.

structural descriptors and the pharmacophoric profiles of M1 and hexahydropyrimidine derivatives. First, both groups of compounds contain saturated *N*-containing heterocycles, aromatic rings, and ester groups (Fig. 5A). Second, both types of compounds have reaction centers in their molecules such as hydrogen bond donors and acceptors capable of hydrogen and salt bridge formation with polar amino acids present in the active site (Fig. 5B). The presence of aromatic rings suggests π-π stacking interactions with tyrosine and tryptophan, functional amino acids.

Meanwhile, compound **1e** surpasses M1 agonists in the number of rotating bonds, which increases the number of degrees of freedom when the molecule is being docked to the active site.

Then for the construction of the regression model, we implemented the induced fit docking (IFD protocol) of M1 receptor agonist to the orthosteric active binding site and plotted the dependence of the docking score on the half-maximal effective concentration (M_1 EC_{50}) determined by the radioligand binding assay [40]. The correlation index of the *in vitro* and *in silico* data exceeded 70%, which validated the docking protocol used. The regression model was verified: the results of docking of the selective M1 agonist, ML071, predict the EC_{50} value of 0.20 μM *versus* the EC_{50} value of 0.19 μM obtained in a biological assay [40].

Lead compounds **1a** and **1e** selected as a result of biological assays were docked to the orthosteric active binding site. The resulting docking scores were used to predict the EC_{50} values

The docking scores for he hexahydropyridine derivatives fall in the range from –7.2 to –10.5, which is typical of M1 agonists (Fig. 6, Table 2). For compound **1e**, the docking score characterizing the ligand affinity to the target is close to this value for the agonist ML071. Considering the predicted EC_{50} values, compound **1e** appears more active towards M1 acetylcholine receptor than compound **1a**.

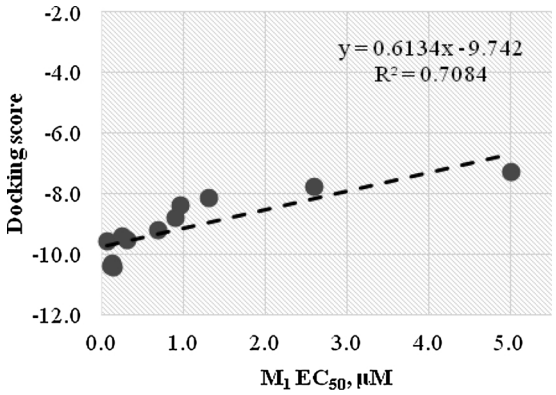

Fig. 6. Regression dependence for the half-maximal effective concentration EC_{50} and docking scores of M1 agonists to the active site.

Table 2
Results of molecular docking to the orthosteric site of the M1-receptor.

ID compound	M_1 EC_{50} (μM) exp	M_1 EC_{50} (μM) calc	Docking score
ML071	0.20	0.19	−9.6
1a	—	4.60	−6.9
1e	—	1.11	−9.1

4. Discussion

Cognitive enhancers are a group of neuropsychotropic drugs with different mechanisms of action that can improve cognitive and intellectual functions not only in patients but also in healthy individuals, which is currently important for both young and elderly persons. The purpose of this study was to assess the effect of new hexahydropyrimidine derivatives on cognitive functions and memory (passive avoidance) and on the behavior in the open field test in intact (without pathology) rats. Studying their effect on the life span of mice under conditions of normobaric hypoxia with hypercapnia and hypoxia caused by toxic substances, as well as an assessment of the antioxidant properties of the identified leading compounds on the background of

toxic hypoxia. In the literature, similar structures exhibiting neurotropic activity are described. *In vitro*, fluorine-containing hexahydropyrimidine derivatives are known to manifest themselves as multifunctional compounds, with cholinesterases and β-secretase as well as β-amyloid inhibitory activities [7]. Dunbar et al. (1993) *in vitro* experiments showed that rat hippocampal sections treated with 1,4,5,6-tetrahydropyrimidine increased phosphoinositide level, the synthesis of which depends on the activation of the M1-muscarine acetylcholine receptor (mAChR) [42]. Further, for a number of derivatives of tetrahydropyrimidine and tetrahydropyridine, the ability to bind to M1, M2, or M3 receptors was expressed in A9 L cells [1] was shown. In an *in vivo* study on intact rats, a 1,4,5,6-tetrahydropyrimidine derivative (CDD-0102A), an M1 mAChR agonist, enhanced both delayed spontaneous alternation and strategy switching [24]. The similarity of the molecular structures of new hexahydropyrimidine derivatives with the structures described earlier in the literature prompted us to begin *in vivo* studies with the identification of the cognitive-enhancement activity of these compounds. The passive avoidance test is a common method for studying biologically active compounds that affect the cognitive functions associated with the limbic system (memory, learning, processing and storage of information) [24,43]. We found that hexahydropyrimidine derivatives single treatment in the phase of input and initial processing of information in the passive avoidance, that four out of five compounds are more or less beneficial for learning process in intact female rats. Among them, the most active derivatives, having in its composition glycine (**1a**) and *l*-tyrosine (**1e**). In the subsequent determination of the average effective dose of the leading compounds on the passive avoidance model, it was shown that both compounds increase the latent period in a dose-dependent manner and reduce the time spent in a dark chamber when the reflex is played after 24 h (Table SM3, SM4). Test OF makes clear the influence of various factors on the behavioral functions associated with the study and fear of unfamiliar space [44–46]. Under OF conditions, the research behavior in females is more pronounced than in males [44,45]. In the OF test, there was no significant effect of hexahydropyrimidine derivatives on the horizontal and vertical locomotor activity of animals, but there was a decrease in the number of grooming episodes in the experimental groups compared with the control. Accoding to N. Sestakova et al. [44] a reduction in the number of grooming episodes is characterized by a decrease in vertical motor activity and a decrease in decision-making time. An increase in the number of grooming episodes indicates an anxiogenic component of research behavior (anxiety-like behavior) in an unfamiliar space during the study of the OF [45,47]. The reduction in the number of grooming episodes in our test may be due to the anxiolytic effect of the derivatives of hexahydropyrimidine and piracetam, which we have chosen as the reference drug [48]. Of greatest interest as biological targets for treating hexahydropyrimidine are the M1 and M4 mAChR subtypes located in the hippocampus and the cerebral cortex, the sites responsible for learning, memory and cognitive activity, and unlike the M2 and M3 mAChR subtypes, have a lesser effect on functional activity of the heart muscle and the gastrointestinal tract [5,6]. In the literature, tetrahydropyrimidine derivatives are shown as muscarinic ligands of M1, M2, M3, M4 acetylcholine receptors [1,24].

Since the effect on the cognitive function may be associated with the influence on the M1 mAChR, it was reasonable to pursue theoretical studies along this line. According to the results of molecular modeling, compounds **1a** and **1e** can be bound in the orthosteric active site by forming a number of intermolecular interactions with functional amino acids. The agonist ML071 and compound **1e** form π-π stacking interactions between the aromatic rings and tyrosines in positions 381, 404, and 408 and tryptophan in position 378. Also, hydrogen bridges are formed between the ester oxygen atoms of the test compounds and hydrogen of asparagine 382. The position of structure **1a** somewhat differs from positions of **1e** and ML071: the molecule is located in the lower part of the active site and forms the π-cation *via* stacking interactions between the protonated nitrogen atom of the heterocycle and aromatic amino acids. In addition, these compounds can act as ligands and M4 mAChR (Fig. SM3).

Since cognitive impairment can be associated with brain hypoxia [2,3], it is of interest to study hexahydropyrimidine derivatives as compounds that affect lipoperoxidation processes in conditions of acute hypoxia. Reference drug piracetam improves cognitive processes, having a multifunctional effect on the central nervous system, including, has a protective effect in violation of brain function due to hypoxia and intoxication [48–50]. On the model of normobaric hypoxia with hypercapnia, we identified two compounds, which we investigated on models of hypoxia caused by toxic substances. On the model of hypoxia caused by sodium nitroprusside, which causes disruption of the mitochondrial electron transport chain, the compounds exhibit antihypoxic properties, reducing the level of MDA and increasing the level of catalase in the brain.

Based on the obtained results, hexahydropyrimidine derivatives seem to us promising compounds that require further research on their effects on cognitive functions on models of pathologies associated with memory damage caused by cholinergic dysfunction and ischemic stroke, with an emphasis on studying the functional activity of mitochondria.

5. Conclusion

Biological assays of hexahydropyrimidine derivatives *in vivo* revealed two compounds that had a pronounced effect on the cognitive function of rodents and showed antihypoxic activity comparable with or exceeding that of piracetam used as the reference agent. According to the molecular docking data, the cognitive enhancement may be associated with the influence of new hexahydropyrimidine derivatives on the M1 muscarinic receptor function.

Declaration of Competing Interest

The authors declare no conflict of interest.

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References

[1] W.S. Messer, Y.F. Abuh, Y. Liu, S. Periyasamy, D.O. Ngur, M.A. Edgar, A.A. El-Assadi, S. Sbeih, P.G. Dunbar, S. Roknich, T. Rho, Z. Fang, B. Ojo, H. Zhang, J.J. Huzl 3rd, P.I. Nagy, Synthesis and biological characterization of 1,4,5,6-tetrahydropyrimidine and 2-amino-3,4,5,6-tetrahydropyridine derivatives as selective M1 agonists, J. Med. Chem. 40 (8) (1997) 1230–1246, https://doi.org/10.1021/jm960467d.

[2] F. Yang, L. Zhou, D. Wang, Z. Wang, Q.-Y. Huang, Minocycline ameliorates hypoxia-induced blood-brain barrier damage by inhibition of HIF-1a through SIRT-3/PHD-2 degradation pathway, Neuroscience 304 (2015) 250–259, https://doi.org/10.1016/j.neuroscience.2015.07.051.

[3] S.A. Adefegha, G. Oboh, O.S. Omojokun, O.M. Adefegha, Alterations of Na^+/K^+-ATPase, cholinergic and antioxidant enzymes activity by protocatechuic acid in

cadmium-induced neurotoxicity and oxidative stress in Wistar rats, Biomed. Pharmacother. 83 (2016) 559–568, https://doi.org/10.1016/j.biopha.2016.07.017.
[4] Z. Zhu, G. Reiser, Signaling mechanism of protease activated receptor 1-induced proliferation of astrocytes: stabilization of hypoxia inducible factor-1α triggers glucose metabolism and accumulation of cyclin D1, Neurochem. Int. 79 (2014) 20–32, https://doi.org/10.1016/j.neuint.2014.09.010.
[5] D.M. Thal, B. Sun, D. Feng, V. Nawaratne, K. Leach, C.C. Felder, M.G. Bures, D.A. Evans, W.I. Weis, P. Bachhawat, T.S. Kobilka, P.M. Sexton, B.K. Kobilka, A. Christopoulos, Crystal structures of the M1 and M4 muscarinic acetylcholine receptors, Nature 531 (7594) (2016) 335–340, https://doi.org/10.1038/nature17188.
[6] W. Froestl, A. Muhs, A. Pfeifer, Cognitive enhancers (nootropics). Part 1: drugs interacting with receptors, J. Alzheimers Dis. 32 (2012) 793–887, https://doi.org/10.3233/JAD-2012-121186.
[7] D. Panek, A. Wieckowska, T. Wichur, M. Bajda, J. Godyn, J. Jonczyk, K. Mika, J. Janockova, O. Soukup, D. Knez, J. Korabecny, S. Gobec, B. Malawska, Design, synthesis and biological evaluation of new phthalimide and saccharin derivatives with alicyclic amines targeting cholinesterases, beta-secretase and amyloid beta aggregation, Eur. J. Med. Chem. 125 (2017) 676–695, https://doi.org/10.1016/j.ejmech.2016.09.078.
[8] A.B. Jha, S.S. Panchal, A. Shah, Ellagic acid: insights into its neuroprotective and cognitive enhancement effects in sporadic Alzheimer´s disease, Pharmacol. Biochem. Behav. 175 (2018) 33–46, https://doi.org/10.1016/j.pbb.2018.08.007.
[9] F.J. Martins, C.A. Caneschi, M.P. Senra, G.S.G. Carvalho, A.D. da Silva, N.R.B. Raposo, In vitro antifungal activity of hexahydropyrimidine derivatives against the causative agents of dermatomycosis, Sci. World J. (2017) 1–8, https://doi.org/10.1155/2017/1207061 id 1207061.
[10] O.C. Agbaje, O.O. Fadeyi, S.A. Fadeyi, L.E. Myles, C.O. Okoro, Synthesis and in vitro cytotoxicity evaluation of some fluorinated hexahydropyrimidine derivatives, Bioorg. Med. Chem. Lett. 21 (2011) 989–992, https://doi.org/10.1016/j.bmcl.2010.12.022.
[11] D. Horvath, A virtual screening approach applied to the search for trypanothione reductase inhibitors, J. Med. Chem. 40 (1997) 2412–2423, https://doi.org/10.1021/jm9603781.
[12] L.V. Azaryan, S.A. Avetisyan, N.S. Buyukyan, A.A. Chachoyan, B.T. Garibdzhanyan, Synthesis and antitumor activity of the 6-p-alkoxyphenyl derivatives of hexahydropyrimidine-2,4-diones, Pharm. Chem. J. 31 (1997) 282–284, https://doi.org/10.1007/BF02464114.
[13] N.N. Gibadullina, D.R. Latypova, V.A. Vakhitov, D.V. Khasanova, L.F. Zainullina, Yu.V. Vakhitova, A.N. Lobov, B.I. Ugrak, Yu.V. Tomilov, V.A. Dokichev, Synthesis and cytotoxic activities of difluoroacetyl-substituted hexahydropyrimidine derivatives, J. Fluor. Chem. 211 (2018) 94–99, https://doi.org/10.1016/j.jfluchem.2018.04.011.
[14] J.H. Billman, J.L. Meisenheimer, Hexahydropyrimidines. Iv. Synthesis of 2-(4-(N,N-bis(2-chloroethyl)amino)aryl)-1,3-bis(aralkyl)hexahydropyrimidines as antitumor agents, J. Med. Chem. 7 (1964) 115–117, https://doi.org/10.1021/jm00331a028.
[15] S. Groszkowski, L. Korzycka, W. Bilasiewicz, 1,3-Di-(halogenoacyl)-piperimidine and 2-methylpiperimidine derivatives, Pol. J. Pharm. Pharm. 25 (1973) 573–577.
[16] G.S. de Carvalho, R.M. Dias, F.R. Pavan, C.Q. Leite, V.L. Silva, C.G. Diniz, D.T. de Paula, E.S. Coimbra, P. Retailleau, A.D. da Silva, Synthesis, cytotoxicity, antibacterial and antileishmanial activities of imidazolidine and hexahydropyrimidine derivatives, Med. Chem. 9 (3) (2013) 351–359, https://doi.org/10.2174/1573406411309030005.
[17] K. Drandarov, A. Guggisberg, M. Ilesse, Macrocyclic spermine alkaloids from Verbascum: the (EIZ)-Isomeric pairs (-)-(S)-verbasitrine/(-)-(S)-isoverbasitrine and (+)-(S)-Verbametrine/(+)-isoverbametrine: isolation, structure elucidation, and synthesis, Helv. Chim. Acta 82 (1999) 229–237, https://doi.org/10.1002/chin.199750225.
[18] J.Y. Hwang, H.-Y. Kim, S. Jo, E. Park, J. Choi, S. Kong, D.-S. Park, J.M. Heo, J.S. Lee, Y. Ko, I. Choi, J. Cechetto, J. Kim, J. Lee, Z. No, M.P. Windisch, Synthesis and evaluation of hexahydropyrimidines and diamines as novel hepatitis C virus inhibitors, Eur. J. Med. Chem. 70 (2013) 315–325, https://doi.org/10.1016/j.ejmech.2013.09.055.
[19] R.R. Shakirov, N.N. Yarmukhamedov, L.I. Vlasova, N.Z. Baibulatova, R.Yu. Khisamutdinova, S.F. Gabdrakhmanova, L.T. Karachurina, N.Zh. Baschenko, Synthesis and antiarrhythmic activity of (1,3-dimethyl-5-nitro-5-hexahydropyrimidinyl)-propionic acid methyl ester, Pharm. Chem. J. 40 (2006) 29–31, https://doi.org/10.1007/s11094-006-0051-5.
[20] F. Janati, M.M. Heravi, A. Mirshokraie, Superparamagnetic iron oxide as an efficient catalyst for the one-pot, solvent-free synthesis of 5,5-disubstituted hexahydropyrimidines and their spiro analogues, J. Chem. 5 (2013) 1–5, https://doi.org/10.1155/2013/214617 id 214617.
[21] S.-W. Liu, J. Jin, C. Chen, J.-M. Liu, J.-Y. Li, F.-F. Wang, Z.-K. Jiang, J.-H. Hu, Z.-X. Gao, F. Yao, X.-F. You, S.-Y. Si, C.-H. Sun, PJS, a novel isocoumarin with hexahydropyrimidine ring from Bacillus subtilis PJS, J. Antibiot. 66 (2013) 281–284, https://doi.org/10.1038/ja.2012.118.
[22] A.Q. Siddiqui, L. Merson-Davies, P.M. Cullis, The synthesis of novel polyamine-nitroimidazole conjugates designed to probe the structural specificities of the polyamine uptake system in A549 lung carcinoma cells, J. Chem. Soc. Perkin Trans. I (1999) 3243–3252, https://doi.org/10.1039/A903293B.
[23] E. Septioglu, M.D. Aytemir, Ü. Çalis, Synthesis and anticonvulsant activity of some new hexahydropyrimidine-2,4-dione derivatives, Arzneimittelforschung 55 (5) (2005) 259–264.
[24] M.E. Ragozzino, S. Artis, A. Singh, T.M. Twose, J.E. Beck, W.S. Messer Jr., The selective M1 muscarinic cholinergic agonist CDD-0102A enhances working memory and cognitive flexibility, J. Pharmacol. Exp. Ther. 340 (3) (2012) 588–594, https://doi.org/10.1124/jpet.111.187625.
[25] N.N. Gibadullina, D.R. Latypova, R.A. Novikov, Y.V. Tomilov, V.A. Dokichev, Reaction of trifluoromethyl 1,3-dicarbonyl compounds with formaldehyde and esters of natural α-aminoacids, Arkivoc IV (2017) 222–235, https://doi.org/10.3998/ark.5550190.p010.003.
[26] D.R. Latypova, A.G. Badamshin, N.N. Gibadullina, N.S. Khusnutdinova, L.F. Zainullina, Y.V. Vakhitova, Y.V. Tomilov, V.A. Dokichev, Synthesis and in vitro cytotoxicity evaluation of some N-substituted α-amino acid derivatives containing a hexahydropyrimidine moiety, Med. Chem. Res. 26 (5) (2017) 900–908, https://doi.org/10.1007/s00044-017-1802-4.
[27] G. Mayer, R. Nitsch, S. Hoyer, Effects of changes in peripheral and cerebral glucose metabolism on locomotor activity, learning and memory in adult male rats, Brain Res. 532 (1990) 95–100, https://doi.org/10.1016/0006-8993(90)91747-5.
[28] N.V. Sayapina, T.A. Batalova, V.V. Chaika, V.L. Kuznetsov, A.A. Sergievich, V.P. Kolosov, Y.M. Perel'man, K.S. Golokhvast, Multi-walled carbon nanotubes increase anxiety levels in rats and reduce exploratory activity in the open field test, Dokl. Biol. Sci. 464 (2015) 223–225, https://doi.org/10.1134/S0012496615050026 doi: 10.1134/S0012496615050026. Epub 2015 Nov 4.
[29] L.V. Shurygina, E.I. Zlishcheva, A.N. Kravtsova, A.A. Kravtsov, Antioxidant and antiamnestic effects of potassium comenate and comenic acid under conditions of normobaric hypoxia with hypercapnia, Bull. Exp. Biol. Med. 163 (3) (2017) 344–348, https://doi.org/10.1007/s10517-017-3800-4.
[30] V.V. Iasnetsov, T.A. Voronina, Antihypoxic and antiamnesic effects of mexidol and semax, Eksp. Klin. Farmakol. 73 (4) (2010) 2–7 In Russian.
[31] A.G. Malykh, M.R. Sadaie, Piracetam and piracetam-like drugs: from basic science to novel clinical applications to CNS disorders, Drugs 70 (3) (2010) 287–312, https://doi.org/10.2165/11319230-000000000-00000.
[32] K. Yagi, Assay for blood plasma or serum, Methods Enzymol. 105 (1984) 328–331, https://doi.org/10.1016/S0076-6879(84)05042-4.
[33] A. Ataie, M. Sabetkasaei, A. Haghparast, A.H. Moghaddam, B. Kazeminejad, Neuroprotective effects of the polyphenolic antioxidant agent, Curcumin, against homocysteine-induced cognitive impairment and oxidative stress in the rat Pharmacology, Biochem. Behav. 96 (2010) 378–385, https://doi.org/10.1016/j.pbb.2010.06.009.
[34] L. Goth, A simple method for determination of serum catalase activity and revision of reference range, Clin. Chim. Acta 196 (1991) 143–152.
[35] M.A. Korolyuk, L.I. Ivanova, I.G. Maiorova, V.E. Tokarev, A method for measuring catalase activity, Lab. Delo 1 (1988) 16–19.
[36] OECD Guidelines for the Testing of Chemicals, Section 4, Test No. 425 Acute Oral Toxicity: Up-and-Down-Procedure, (2008), p. 27, https://doi.org/10.1787/9789264071049-en.
[37] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The protein data bank, Nucleic Acids Res. 28 (2000) 235–242.
[38] E. Harder, W. Damm, J. Maple, C. Wu, M. Reboul, J.Y. Xiang, L. Wang, D. Lupyan, M.K. Dahlgren, J.L. Knight, J.W. Kaus, D.S. Cerutti, G. Krilov, W.L. Jorgensen, R. Abel, R.A. Friesner, OPLS3: a force field providing broad coverage of drug-like small molecules and proteins, J. Chem. Theory Comput. 12 (1) (2016) 281–296, https://doi.org/10.1021/acs.jctc.5b00864.
[39] L.J. Henderson, Concerning the relationship between the strength of acids and their capacity to preserve neutrality, Am. J. Physiol. 21 (4) (1908) 173–179, https://doi.org/10.1152/ajplegacy.1908.21.2.173.
[40] E.P. Lebois, G.J. Digby, D.J. Sheffler, B.J. Melancon, J.C. Tarr, H.P. Cho, N.R. Miller, R. Morrison, T.M. Bridges, Z. Xiang, J.S. Daniels, M.R. Wood, P.J. Conn, C.W. Lindsley, Development of a highly selective, orally bioavailable and CNS penetrant M1 agonist derived from the MLPCN probe ML071, Bioorg. Med. Chem. Lett. 21 (2011) 6451–6455, https://doi.org/10.1016/j.bmcl.2011.08.084.
[41] E.P. Lebois, T.M. Bridges, E.S. Dawson, Jp. Kennedy, Z. Xiang, S.B. Jadhav, H. Yin, J. Meiler, C.K. Jones, P.J. Conn, C.D. Weaver, C.W. Lindsley, ACS Chem. Neurosci. 1 (2010) 104.
[42] P.G. Dunbar, G.J. Durant, Zh. Fang, Y.F. Abuh, M.A. El-Assadi, D.O. Ngur, S. Periyasamy, W.P. How, W.S. Messer Jr., Design, synthesis, and neurochemical evaluation of 5-(3-Alkyl-1,2,4-oxadiazol-5-yl)-1,4,5,6-tetrahydropyrimidinaess MI muscarinic receptor agonists, J. Med. Chem. 36 (1993) 842–847.
[43] K. Laszlo, K. Toth, E. Kertes, L. Peczely, T. Ollmann, A. Madarassy-Szucs, L. Lenard, The role of neurotensin in passive avoidance learning in the rat central nucleus of amygdala, Behav. Brain Res. 226 (2012) 597–600, https://doi.org/10.1016/j.bbr.2011.08.041.
[44] N. Sestakova, A. Puzserova, M. Kluknavsky, I. Bernatova, Determination of motoractivity andanxiety-related behavior in rodents: methodological aspects and role of nitricoxide, Interdiscip. Toxicol. 6 (3) (2013) 126–135, https://doi.org/10.2478/intox-2013-0020).
[45] A. Muntsant, K. Shrivastava, M. Recasens, L. Giménez-Llort, Severe perinatal hypoxic-ischemic brain injury induces long-term sensorimotor deficits, anxiety-like behaviors and cognitive impairment in a sex-, age- and task-selective manner in C57BL/6 mice but can be modulated by neonatal handling, Front. Behav. Neurosci. 13 (2019) 7, https://doi.org/10.3389/fnbeh.2019.00007 www.frontiersin.org.

[46] K.H. Struntz, J.A. Siegel, Effect of methamphetamine exposure on anxiety-like behavior in the open field test, corticosterone, and hippocampal tyrosine hydroxylase in adolescent and adult mice, Behav. Brain Res. 348 (2018) 211–218, https://doi.org/10.1016/j.bbr.2018.04.019.
[47] D.E. Hendricks, B. Seaythe, Open field as a test of emotionality, Behav. Res. Meth. Instrum. 5 (1) (1973) 61.
[48] S.K. Bhattacharya, A.P. Sen, S.N. Upadhyay, A.K. Jaiswal, Anxiolytic activity of piracetam, a nootropic agent, following subchronic administration in rodents, Indian J. Exp. Biol. 31 (11) (1993) 902–907.
[49] U. Keil, A. Bonert, C.A. Marques, I. Scherping, et al., Amyloid beta–induced changes in nitric oxide production and mitochondrial activity lead to apoptosis, J. Biol. Chem. (2004) 279–285.
[50] H.S. Foyet, H.H.N. Abaïssou, E. Wado, E.A. Acha, C. Alin, Emilia coccinae (SIMS) G Extract improves memory impairment, cholinergic dysfunction, and oxidative stress damage in scopolamine treated rats, BMC Complement. Altern. Med. 15 (2015) 333, https://doi.org/10.1186/s12906-015-0864-4.