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Article

Ex Vivo and In Vivo Study of Some Isoquinoline Precursors

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Abstract: This article concerns the synthesis and biological activities of some N-(1-(3,4-dimethoxyphen yl)propan-2-yl) amides as isoquinoline precursors and compounds with smooth muscle (SM) relaxant activity. Aim: find the biological activity of N-(1-(3,4-dimethoxyphenyl)propan-2-yl) amides and compare it with papaverine, an isoquinoline alkaloid that has been known as a brain and coronary vasodilator and SM relaxant. Materials and methods: In silico simulation with the PASS online program predicts SM relaxant activity for the compounds. The amides were tested on the isolated gastric SM preparations (SMPs) from rats to determine their effects on spontaneous contractile activity (CA) compared with papaverine. The in vivo effect on the learning and memory processes of rats was also assessed. Results: the data from the isometric measurements showed that one of the compounds caused ex vivo relaxation in circular SM tissues isolated from the stomach (corpus) of male Wistar rats. Conclusion: We found that the compound's SM relaxation uses the papaverine pathway. It also has an improving effect on the cognitive functions of learning and memory processes in rats.

Keywords: synthesis; isoquinoline precursors; in silico; smooth muscle relaxants; learning and memory processes in rats

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1. Introduction

Isoquinolines are a family of phytochemicals found in plants, such as Papaveraceae, Berberidaceae, and Ranunculaceae. Alkaloids found in these plants show a remarkable number of biological activities. The isoquinoline ring has been found to possess a wide range of biological and pharmacological activities such as antimalarial, anti-HIV, insect growth retardant, antitumor, antimicrobial, antibacterial, etc., [1]. One well-known isoquinoline is papaverine (Figure 1); it belongs to the benzylisoquinoline alkaloid group and for decades has been used as a brain and coronary vasodilator, muscle relaxant, as well as for its non-specific spasmolytic activity [2]. Papaverine is also a potent, specific inhibitor of phosphodiesterase 10A (PDE10A) and has been reported to increase cognitive performance in a rat model of schizophrenia [3].

The vasodilation effect of papaverine has been credited to the inhibition of cyclic nucleotide phosphodiesterases (PDEs), with resulting rises in intracellular levels of the cyclic AMP and cyclic GMP accompanied by declines in Ca^{2+}. This alkaloid produces a prolonged myocardial refractory period by decreasing the conduction rate [4–7].

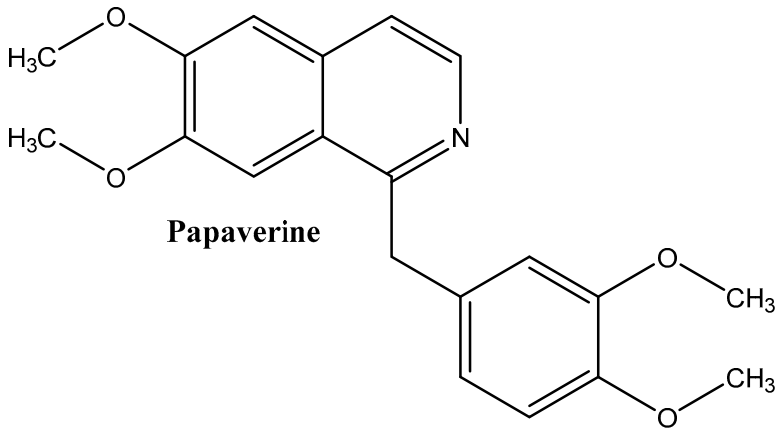


Figure 1. Chemical structure of papaverine.

Some natural benzylisoquinoline alkaloids have a relaxation effect on SMs [8], including abnormal contraction preliminary caused by KCl depolarization [9]. This makes them good new drugs with potential relaxing properties, similar to known ones, such as papaverine and drotaverine. Isoquinoline alkaloids also have antidepressant properties affecting serotonin mediation [10], as well as a cytoprotective effect [11,12]. Isoquinolines and tetrahydroisoquinolines can serve as good anesthetics, vasodilators, and antihypertensive and antibacterial agents [13]. These compounds possess an inotropic effect through their influence on Ca^{2+} modulation [14] and have significant protective activity on memory dysfunction in rats, as well as cholinesterase (ChE) inhibitor activity [15]. The relationship between drugs inhibiting PDE activity and SM relaxation in the isolated guinea-pig colon has been well-known for a long time [16,17].

The decline of cognitive function is associated not only with the natural aging process, but also accompanies several CNS diseases such as various types of dementia, depressive disorders, schizophrenia, and epilepsy. The social significance of the problem requires the development of new strategies for the therapeutic impact of learning and memory processes.

Cholinesterase inhibition remains a leading position in the treatment of cognitive impairments observed in Alzheimer's dementia [18]. The role of serotonergic and monoaminergic neurotransmitter systems has been studied [19,20]. The importance of PDEs and their inhibition of learning and memory processes has been less investigated.

From this point of view, isoquinolines can be an important starting point for drug discovery. Using the isoquinoline structure as a base model, we investigated the synthesis of some starting compounds for isoquinoline ring synthesis as isoquinoline precursors and compounds having SM relaxant activity.

The aforementioned studies show us a perspective to investigate CA and learning and memory processes in rats for the synthesized compounds.

2. Materials and Methods

2.1. Synthetic Methods

All solvents and reagents were purchased from Merck and Sigma-Aldrich. Melting points were determined on a Boetius hot-stage apparatus and are uncorrected. All the compounds were characterized by ^{1}H NMR, ^{13}CNMR, IR, and microanalysis. The purity of these compounds was determined by TLC using several solvent systems of different polarity. TLC was carried out on precoated 0.2 mm Fluka silica gel 60 plates (Merck KGaA, Darmstadt, Germany), using chloroform: diethyl ether: n-hexane = 6:3:1 as a chromatographic system. Elemental analyses were performed with a TruspecMicro (LECO, Mönchengladbach, Germany). Neutral Al_2O_3 was used for column chromatographic separation. The products, after evaporation of the solvent, were purified by recrystallization from diethyl ether.

IR spectra were determined on a VERTEX 70 FT-IR spectrometer (Bruker Optics, Ettlingen, Germany). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 500 spectrometer (Bruker, Billerica, MA, USA) at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR), respectively. Chemical shifts are provided in relative ppm and were referenced to tetramethylsilane (TMS) (δ = 0.00 ppm) as an internal standard; the coupling constants are indicated in Hz. The NMR spectra were recorded at room temperature (ac. 295 K). Liquid chromatography with mass detection (LC-MS/MS) of analytes was performed using the chromatographic system Thermo Dionex Ultimate 3000 LC and triple quadrupole mass spectrometer Thermo TSQ Quantum Access MAX (Thermo Fisher Scientific, Waltham, MA, USA), with Heated Electrospray Ionization (HESI). The chromatographic system includes a quaternary two-piston pump, autosampler, and column thermostat. Chromatographic separation was performed under isocratic conditions on a core shell analytical column Accucore^{TM} RP-MS with Length 100 mm, diameter 2.1 mm, and 2.6 µm particles size (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic conditions were established in an isocratic mode for sample analysis. The peak shapes and MS signals of the analytes were improved by using mobile phases A: 0.1% formic acid in acetonitrile-water (90:10, *v*/*v*); and B: 0.1% formic acid in acetonitrile-water (10:90, *v*/*v*) in 40:60 (A:B) ratio at flow rate 0.150 mL min^{-1}. HESI was used for analyte detection in positive ionization mode with spray voltage −4000 V; source temperature 400 °C; sheath gas pressure 30; vaporizer temperature 350 °C; capillary temperature 270 °C. Protonated molecules of analyte were used as precursor ions for selected reaction monitoring (SRM).

2.1.1. Synthesis of 1-(3,4-Dimethoxyphenyl)propan-2-amine **3**

To a solution of 5 mmol of the starting ketone 1-(3,4-dimethoxyphenyl)propan-2-one **1** in 25 mL formamide, a catalytic amount of methanoic acid was added. The mixture was refluxed for 2 h at 180 °C, then poured in water and extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were washed with Na_2CO_3 solution, water, and dried using anhydrous Na_2SO_4, filtered on the short column filled with neutral Al_2O_3, and then concentrated.

To a solution of 5 mmol of N-(1-(3,4-dimethoxyphenyl)propan-2-yl)formamide **2**, 50 mL 5N H_2SO_4 was added. The mixture was refluxed for 1 h at 100 °C, then poured in water and extracted with CH_2Cl_2 (2 × 20 mL). The water layer was alkalized with NH_4OH and extracted with CH_2Cl_2 (3 × 20 mL). The combined extracts were dried using anhydrous Na_2SO_4, filtered on the short column filled with basic Al_2O_3, and then concentrated.

The following was performed: 1-(3,4-dimethoxyphenyl)propan-2-amine (**3**): ^{1}H-NMR: 1.17 (d, J = 6.1, 3H, CH-CH_3), 2.03 (broad s, 2H, NH_2), 2.48–2.56 (m, 1H, CH_2), 2.67–2.74 (m, 1H, CH_2), 3.16–3.21 (m, 1H, CH-CH3), 3.88 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.74–6.77 (m, 2H, Ar), 6.82–6.85 (m, 1H, Ar) (Figure S1); ^{13}C-NMR: 148.9, 147.6, 121.2, 112.5, 111.4, 55.95, 55.9, 48.6, 45.8, 23.2 (Figure S2). Anal. calcd. for $C_{11}H_{17}NO_2$: C, 67.66; H, 8.78; N, 7.17; found: C, 67.67; H, 8.75; N, 7.18.

2.1.2. Preparation of 1-(3,4-Dimethoxyphenyl)propan-2-amides **4**; Typical Procedure

To a solution of 3 mmol 1-(3,4-dimethoxyphenyl)propan-2-amine **3**, 3.5 mmol of the corresponding acyl chloride in dichloromethane (10 mL) was added. Then 3.4 mmol $N(C_2H_5)_3$ was added after 10 min. After approximately 30 min the reaction mixture was washed consequently with diluted HCl (1:4), Na_2CO_3, and H_2O, then dried with anhydrous Na_2SO_4, filtered on the short column filled with neutral Al_2O_3, and concentrated.

See the following: N-(1-(3,4-dimethoxyphenyl)propan-2-yl)acetamide (**4a**): mp = 83–85 °C, 81% yield, ^{1}H-NMR: 1.11 (d, J = 6.8, 3H, CH-CH_3), 2.50 (s, 3H, $COCH_3$), 2.64 (dd, J = 13.7, 6.8, 1H, CH_2), 2.8 (dd, J = 13.7, 5.9, 1H, CH_2), 3.86 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 4.22–4.24 (m, 1H, CH-CH_3), 5.36 (d, J = 6.8, 1H, NH), 6.70–6.79 (m, 2H, Ar), 6.8 (d, J = 8.8, 1H, Ar) (Figure S3); ^{13}C-NMR: 169.3, 148.9, 147.9, 130.5, 121.4, 112.5, 111.1, 55.9, 46.2, 42.02, 23.5, 19.98 (Figure S4); IR(KBr) ν_{max}, cm^{-1}: 3314 v(N–H, >NH), 2966 v(C–H, –CH_3),

2929 v(C–H, >CH_2), 1643 v(C=O), 1519 v(C–C=C, Ph), δ(>CH_2), 1372 δ(–CH_3) (Figure S5); Anal. calcd. for $C_{13}H_{19}NO_3$: C, 65.80; H, 8.07; found: C, 65.77; H, 8.12; N, 5.88.

See the following: N-(1-(3,4-dimethoxyphenyl)propan-2-yl)benzamide (**4b**): mp = 103–104 °C, 80% yield, ^{1}H-NMR: 1.23 (d, J = 6.8, 3H, CH-CH_3), 2.76–2.82 (m, 1H, CH_2), 2.88–2.92 (m, 1H, CH_2), 3.81 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.44–4.46 (m, 1H, CH-CH_3), 6.01 (d, J = 7.8, 1H, NH), 6.74–6.80 (m, 2H, Ar), 6.81–6.82 (m, 1H, Ar), 7.32–7.42 (m, 2H, Ar), 7.46–7.49 (m, 1H, Ar), 7.71 (d, J = 6.8, 2H, Ar) (Figure S6); ^{13}C-NMR: 166.8, 148.9, 147.7, 134.8, 131.4, 130.4, 126.8, 121.6, 112.6, 111.2, 55.9, 55.8, 46.5, 41.9, 19.95 (Figure S7); IR(KBr) ν_{max}, cm^{-1}: 3324 v(N–H, >NH), 2957 v(C–H, –CH_3), 2929 v(C–H, >CH_2), 1637 v(C=O), 1521 v(C–C=C, Ph), δ(>CH_2), 1353 δ(–CH_3) (Figure S8); Anal. calcd. for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.07; N, 4.68; found: C, 72.27; H, 7.09; N, 4.70.

See the following: N-(1-(3,4-dimethoxyphenyl)propan-2-yl)-2-phenylacetamide (**4c**): mp = 120–123 °C, 90% yield, ^{1}H-NMR: 1.06 (d, J = 6.8, 3H, CH-CH_3), 2.57–2.67 (m, 2H, CH_2), 3.50 (s, 2H, CH_2-C_6H_5), 3.81 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.19–4.24 (m, 1H, CH-CH_3), 5.24 (d, J = 7.8, 1H, NH), 6.52 (dd, J = 8.8, 2, 1H, Ar), 6.60–6.61 (m, 1H, Ar), 6.71 (d, J = 7.8, 1H, Ar), 7.14–7.15 (m, 2H, Ar), 7.26–7.33 (m, 3H, Ar) (Figure S9); ^{13}C-NMR: 170.2, 148.8, 147.6, 134.9, 130.3, 129.4, 128.95, 127.2, 121.3, 112.4, 111.1, 55.9, 55.8, 46.2, 44.0, 41.89, 20.1 (Figure S10); IR(KBr) ν_{max}, cm^{-1}: 3297 v(N–H, >NH), 2970 v(C–H, –CH_3), 2928 v(C–H, >CH_2), 1636 v(C=O), 1518 v(C–C=C, Ph), δ(>CH_2), 1357 δ(–CH_3) (Figure S11); Anal. calcd. for $C_{19}H_{23}NO_3$: C, 72.82; H, 7.40; N, 4.47; found: C, 72.87; H, 7.45; N, 4.50.

See the following: 2-chloro-N-(1-(3,4-dimethoxyphenyl)propan-2-yl)-2-phenylacetamide (**4d**): mp = 87–89 °C, 70% yield, ^{1}H-NMR: 1.24 (d, J = 6.8, 3H, CH-CH_3), 2.75–2.79 (m, 1H, CH_2), 2.92 (dd, J = 13.7, 6.8, 1H, CH_2), 3.85 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.43–4.46 (m, 1H, CH-CH_3), 6.06 (d, J = 7.8, 1H, NH), 6.77 (s, 1H, CH-Cl), 6.75–6.81 (m, 3H, Ar), 7.28–7.37 (m, 4H, Ar), 7.54–7.56 (m, 1H, Ar) (Figure S12); ^{13}C-NMR: 166.5, 148.9, 147.7, 135.4, 131.1, 130.6, 129.9, 127.0, 121.6, 112.5, 111.2, 61.9, 55.9, 55.8, 47.0, 42.1, 19.95 (Figure S13); IR(KBr) ν_{max}, cm^{-1}: 3330 v(N–H, >NH), 2965 v(C–H, –CH_3), 2924 v(C–H, >CH_2), 1639 v(C=O), 1518 v(C–C=C, Ph), δ(>CH_2), 1375 δ(–CH_3) (Figure S14); HESI m/z 347.96. Transition 347.96 → 179.05 was observed at collision energy 14 V.

See the following: 2-chloro-N-(1-(3,4-dimethoxyphenyl)propan-2-yl)benzamide (**4e**): mp = 88–90 °C, 91% yield, ^{1}H-NMR: 1.24 (d, J = 6.8, 3H, CH-CH_3), 2.75–2.79 (m, 1H, CH_2), 2.91–2.94 (m, 1H, CH_2), 3.86 (s, 6H, OCH_3), 4.45–4.48 (m, 1H, CH-CH_3), 6.03 (d, J = 7.8, 1H, NH), 6.76–6.81 (m, 3H, Ar), 7.27–7.32 (m, 2H, Ar), 7.33–7.38 (m, 2H, Ar), 7.55–7.57 (m, 1H, Ar) (Figure S15); ^{13}C-NMR: 165.8, 148.9, 147.7, 131.2, 129.95, 127.1, 121.6, 112.5, 111.2, 55.9, 55.88, 47.0, 42.0, 19.95 (Figure S16); IR(KBr) ν_{max}, cm^{-1}: 3293 v(N–H, >NH), 2965 v(C–H, –CH_3), 2923 v(C–H, >CH_2), 1634 v(C=O), 1514 v(C–C=C, Ph), δ(> CH_2), 1369 δ(–CH_3) (Figure S17); Anal. calcd. for $C_{18}H_{21}NO_3$: C, 72.22; H, 7.07; N, 4.68; found: C, 72.27; H, 7.05; N, 4.60.

2.2. Smooth Muscle Activity

2.2.1. Ex Vivo Experiments on Gastric SMPs from Rat Wistar

SMPs with dimensions 1.0–1.5 mm wide and 10–15 mm long were obtained from adult male Wistar rats weighing approximately 270 g. Tissues must remain in the solution after dissection to prevent loss of tissue viability, so during the dissection, the tissues were systematically moistened with a solution (NaCl:KCl:$CaCl_2$ in ratio 27.2:1.1:1) prepared at 10 °C. The samples were circularly dissected from corpus gastric muscle and mounted in a tissue bath. One of the hooks on the tissue preparation was tied to the peg on the glass rod and the other one was tied to a tenso detector. Then the samples were superfused with warmed (37 °C) Krebs solution. The pH of the solutions (pH = 7.4; 37 °C) was measured before each experiment by pH-meter HI5521 (Hanna Instruments Inc., Woonsocket, RI, USA) and continuously aerated with a mixture of 95% O_2 and 5% CO_2. Krebs contained the following (in mmol/L): NaCl 120; KCl 5.9; $CaCl_2$ 2.5; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $NaHCO_3$

15.4; glucose 11.5 at pH 7.4. The high-KCl depolarizing solution is similar to Krebs solution but with an equimolar substitution of NaCl with KCl.

2.2.2. Method of Studying a Mechanical Activity of Isolated SM Preparation

The CA of the SMPs and the changes in substance-evoked reactions were detected isometrically by tenso detectors (Swema, Farsta, Sweden) and a Linseis recorder (Linseis Messgeraete GmbH, Selb, Germany). The initial mechanical stress of the preparations obtained by the stretch tension system corresponded to a tensile force of 10 mN.

We equilibrated the tissue for 60 min and during this phase, we washed the tissue every 15–20 min by replacing Krebs solution. In the meanwhile, the compounds were prepared for the experiment. This requires a more concentrated solution than the actual concentration in the bath, so only a small volume (1/100) of the drug stock was needed to achieve the desired concentration. Then, we picked an agonist (a compound that causes active contraction) to which the tissue responds. The intactness of the contractile apparatus of SMPs during and at the end of experiments was checked by adding 1×10^{-6} mol/L ACh between each treatment with drugs. Dose-response data were obtained noncumulatively starting with the lowest dose.

2.3. In Vivo Methods of Studying Cognitive Functions Learning and Memory

The male Wistar rats (170–190 g b.w., 8 per group) were used for studying the effect on learning and memory processes. They were divided into four groups and treated orally once daily: 1st group (control), Dimethyl sulfoxide (DMSO) 0.1 mg/100 g; 2nd group, with a dose of 5 mg/kg; 3rd group, 10 mg/kg; and 4th group, 20 mg/kg from the tested compound. The tests were performed using originally-fabricated apparatus, automatic reflex conditioners for active and passive avoidance (Ugo Basile, Italy). The animals were tested 60 min after drug application.

2.3.1. Shuttle-Box Active Avoidance Test

The learning session was performed for 5 consecutive days (30 trainings daily). Parameters: 6 s light and buzzer 670 Hz and 70 dB, 3 s 0.4 mA foot shock, and 12 s pause. The memory retention test was performed on the 12th day with the same parameters without foot shock.

Automatically counted were the number of conditioned stimuli responses (avoidances), number of unconditioned stimuli responses (escapes from foot shocks), and number of intertrial crossings.

2.3.2. Step-Down Passive Avoidance Test

The learning session was conducted on 2 consecutive days (2 trainings daily with intervals of 60 min). Parameters: 10 s 0.4 mA foot shock. The memory retention test was performed on the 6th day with the same parameters without foot shock. The learning criteria was the latency of reactions with 60 s on the plastic platform.

2.4. Housing and Nutrition

All experimental animals were housed under standard laboratory conditions (23–25 °C, 50–55% humidity, 12/12-h light/dark cycle), while food and water were provided ad libitum.

2.5. Ethics Statement

Animals used in experiments were male Wistar rats. The experiments were approved by the Ethical Committee of the Bulgarian Food Agency with No. 229/09.04.2019 and were carried out following the guidelines of the European Directive 2010/63/EU. Animals were provided by the Animal House of Medical University of Plovdiv, Bulgaria.

2.6. Statistical Analysis

The Instat computer program for analysis of the variance was used. The mean and standard error of mean (SEM) values for each group were calculated. A two-way ANOVA for repeated measurements was used to compare different groups with the respective controls. The *p*-value of $p < 0.05$ was considered representative of a significant difference.

3. Results

Isolated tissue bath assays are a classical pharmacological tool for evaluating concentration-response relationships in a myriad of contractile tissues. Despite this technique being implemented for over 100 years, its versatility, simplicity, and reproducibility remain an indispensable tool for pharmacologists and physiologists [21]. The isolated tissue bath remains an important facet of drug development and basic research as it allows the tissue to function as a tissue. The primary advantage of this technique is that the tissue is living and functions as a whole tissue, with a physiological outcome (contraction or relaxation) that is relevant to the body. Another advantage is that retaining tissue function permits calculation of important pharmacological variables that are more meaningful in a tissue vs. a cellular setting; it comes closer to how the drugs examined would work in the body as a whole [21]. Previously [22–26], the isolated tissue bath was applied for ex vivo SM activity investigation of different compounds, including isoquinoline derivatives. Thus, we applied the same technique to the technique reported in this article with N-(1-(3,4-dimethoxyphenyl)propan-2-yl) amides.

3.1. Effects of **4d** *on Smooth Muscle Activity*

3.1.1. Mutual Influence between Papaverine and **4d**

Simultaneous or single exogenous administration of 1×10^{-6} mol/L papaverine and 5×10^{-5} mol/L of **4d** cause differences in potency and character SM relaxation reactions. The preliminary treatment of SMPs with **4d** and subsequent interaction with papaverine cause a significantly reduced but partially preserved relaxation response to papaverine. When interacting with **4d** following an initial papaverine administration, the **4d**-induced relaxation response disappears completely (Figures 2 and 3a).

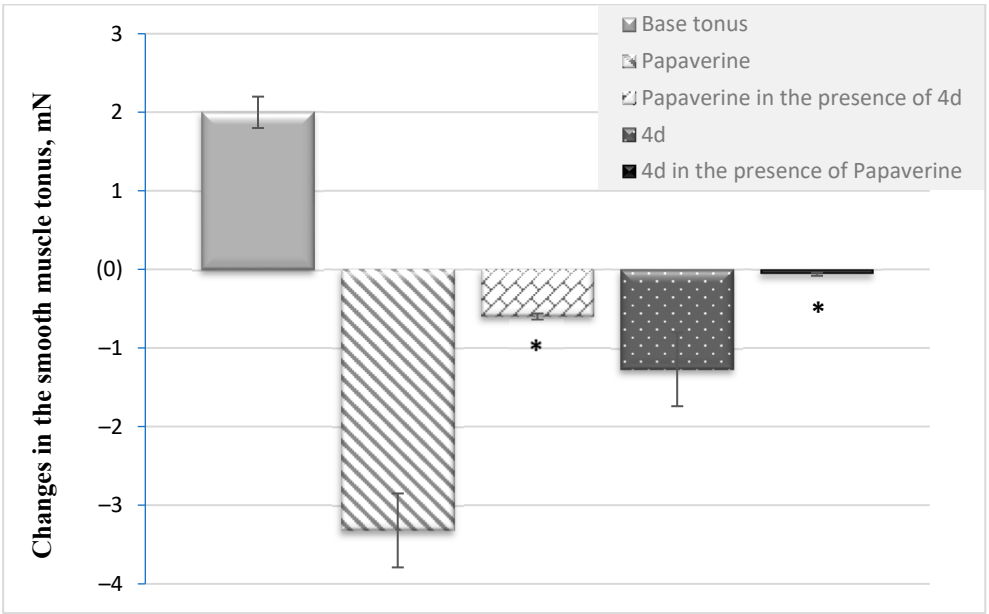


Figure 2. Relaxation effects caused by 1×10^{-6} mol/L papaverine and 5×10^{-5} mol/L **4d** on SMPs at single or combined administration (n = 12); * $p < 0.05$.

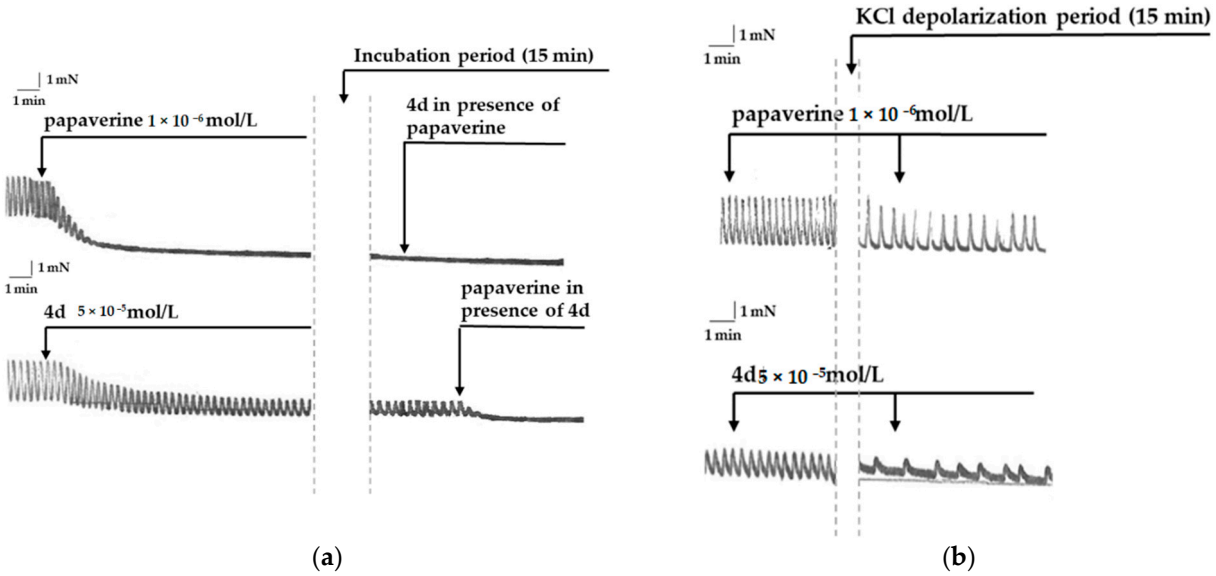


Figure 3. Representative tracings of: (**a**) corpus SMP from rat elicited by 1×10^{-6} mol/L papaverine and 5×10^{-5} mol/L **4d** at single or combined administration; (**b**) SM activity parameters before and after KCl depolarization.

3.1.2. Smooth Muscle Effects of **4d** and Papaverine in Conditions of KCl Depolarization

Replacing regular Krebs solution in the tissue bath with Krebs solution containing 42×10^{-3} mol/L KCl caused initial sudden contractions of the muscle preparations (for approximately 2 min). KCl depolarization allowed obtaining depolarization of the cell membranes as well as activation of the voltage-gated calcium channels. This was followed by stabilization of the muscle tone. The effect caused by **4d** significantly decreased the strength of the tonic component with up to 58.96% ($p < 0.05$) in Krebs solution with increased content of K+ compared with the same test in regular Krebs solution.

The frequency and amplitude of spontaneous CA in the **4d** effect were significantly different; with papaverine, the changes in the relaxation reaction parameters were smaller and statistically insignificantly differentiable ($p > 0.05$) (Table 1, Figure 3b).

Table 1. Changes in the parameters of the spontaneous CA of SMPs when exposed to **4d** (5×10^{-5} mol/L) and papaverine (1×10^{-6} mol/L) in Krebs solution with 42×10^{-3} mol/L KCl. The comparison is between the respective tone, frequency, and amplitude of the CA under the action of **4d** and papaverine in the base Krebs solution.

Applied Agent after KCl Depolarization	SM Activity Parameters	Base Values	% Change	*n*	*p*
4d	Tone	−1.35 mN	58.96	11	0.032
	Frequency	3.19 min^{-1}	47.33		0.012
	Amplitude	0.05 mN	52.61		0.042
Papaverine	Tone	−3.57	81.21	10	0.070
	Frequency	2.9 min^{-1}	85.55		0.057
	Amplitude	0.03 mN	80.09		0.060

3.2. Effects of **4d** *on Cognitive Functions Learning and Memory in Rats*

3.2.1. Shuttle-Box Active Avoidance Test

In the active avoidance test, the control rats produced an increased number of avoidances by days 3, 4, and 5 of the learning session ($p < 0.05$), and day 12 (memory retrieval test) ($p < 0.05$) compared with day 1 (Figure 4).

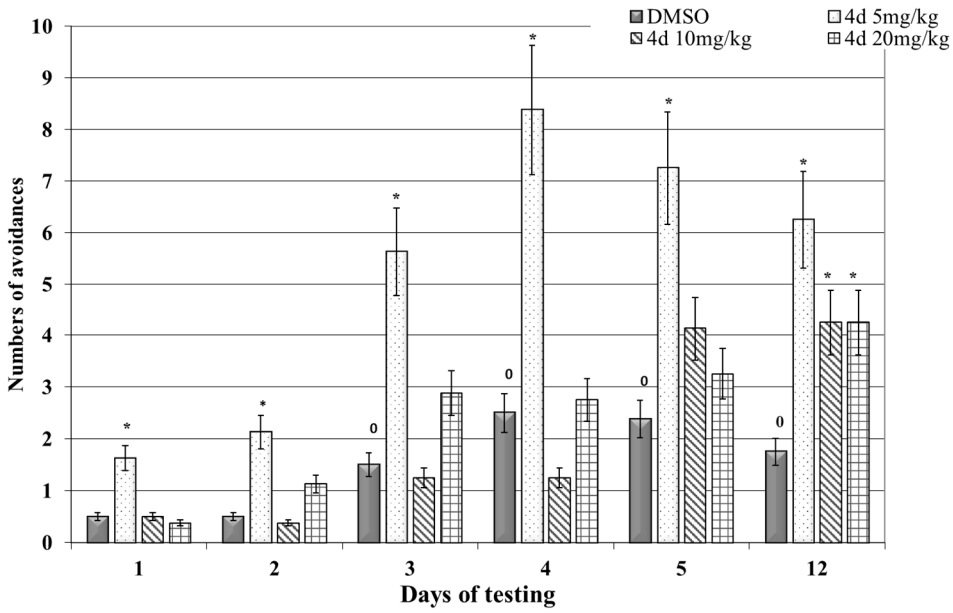


Figure 4. Effects of **4d** on learning and memory processes in rats (shuttle-box active avoidance test, number of avoidances); ^{0} $p < 0.05$ compared with day 1 control group; * $p < 0.05$ compared with the same-day control group.

The animals treated with **4d** at a dose of 5 mg/kg showed an increased number of avoidances during all 1–5 days of the learning session ($p < 0.05$) and in the memory retention test ($p < 0.05$) compared with the same-day control. The groups treated with **4d** at a dose of 10 mg/kg and 20 mg/kg did not change the number of conditioned stimuli responses during the learning session compared with the control of the corresponding days. An increased number of avoidances was recorded in the memory test ($p < 0.05$) compared with the day 12 control group (Figure 4).

In the shuttle-box active avoidance test the control group, treated orally with DMSO, did not change the number of escapes in the learning and memory tests compared with day 1 (Figure 5).

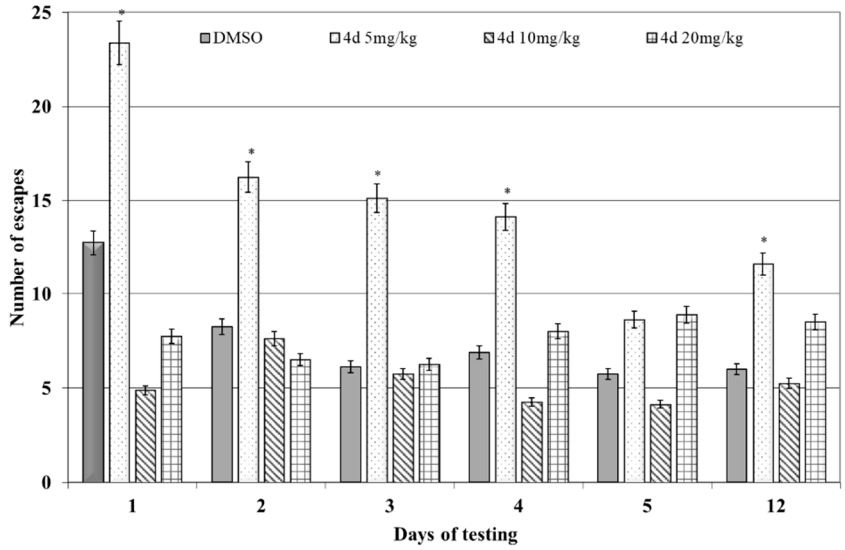


Figure 5. Effects of **4d** on learning and memory processes in rats (shuttle-box active avoidance test, number of escapes) * $p < 0.05$ compared with the same-day control group.

The rats treated with a dosage of 5 mg/kg of **4d** significantly increased the number of unconditioned stimuli responses on days 1, 2, 3, and 4 of learning ($p < 0.05$) and on the

memory retention test ($p < 0.05$) compared with the same-day control. The rats treated with 10 mg/kg and 20 mg/kg of **4d** showed similar to the control group number of escapes on the corresponding days (Figure 5).

In the active avoidance test, the control rats showed no significant change in the number of intertrial crossings during the 5-day learning or in the memory retention sessions, compared with the first-day control (Figure 6).

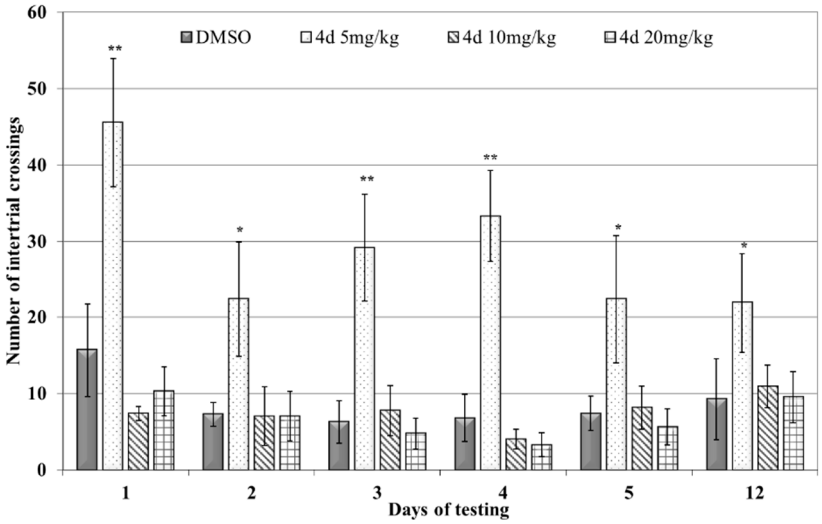


Figure 6. Effects of **4d** on learning and memory processes in rats (shuttle-box active avoidance test, number of intertrial crossings) * $p < 0.05$ compared with the same-day control group; ** $p < 0.01$ compared with the same-day control group.

The animals treated with **4d** at a dose of 5 mg/kg showed an increased number of intertrial crossings on days 1, 3, and 4 ($p < 0.01$), days 2 and 5 ($p < 0.05$) and in the memory retention test on day 12 ($p < 0.05$) compared with the same-day controls (Figure 5). The rats treated with the two larger doses of **4d** exhibited a number of intertrial crossings close to that of the respective day of the control group (Figure 6).

3.2.2. Step-Down Passive Avoidance Test

In the step-down test for passive learning, the control rats did not change the latency of reactions in the learning test but significantly increased it in the memory retention test ($p < 0.05$) compared with day 1 (Figure 7).

We found that animals with **4d** at a dose of 5 mg/kg increased the latency of reactions on days 1 and 2 of the learning session ($p < 0.05$) compared with the same-day controls. The groups with **4d** at a dose of 10 mg/kg or 20 mg/kg did not change the latency of reactions in the 2-day learning compared with the corresponding day of the control group. In the memory retention test, the groups treated with **4d** at a dose of 5 mg/kg or 10 mg/kg increased the latency time compared with the same-day of the controls, but it was not statistically significant.

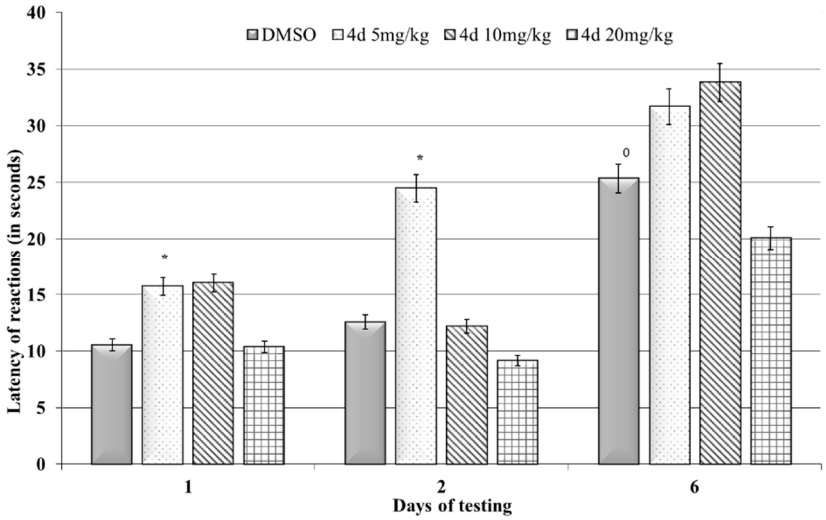


Figure 7. Effects of **4d** on learning and memory processes in rats (step-down passive avoidance test); ^{0} $p < 0.05$ compared with day 1 control group; * $p < 0.05$ compared with the same-day control group.

4. Discussion

Studying the biological activity of newly synthesized molecules is a relevant scientific matter. This is related to the unfaltering interest in discovery, and in this case, of a molecule with specific "useful" characteristics, which is capable of affecting the activity of particular structures and through them, processes in the human body.

In our previous reports we discussed synthesis of 2-chloro-N-((S)-1-(3,4-dimethoxyphenyl)propan-2-yl)-2-phenylacetamide (IQP) from starting alanine and its bioelectrical activity on SM [20]. There are two possible pathways for relaxation of SM: influence of the Ca^{2+} channels and reducing Ca^{2+} influx, and impact on the intracellular NO. We showed the influence of IQP on the L-type Ca^{2+} channels [27]. We concluded that SM relaxation is provoked by the effect of IQP on the Ca^{2+} influx towards SM, an effect that leads to decreased intracellular Ca^{2+} levels. We found that when administering the compound in a range of $7.5 \times 10^{-6} \div 2.5 \times 10^{-4}$ mol/L on the isolated gastric SMPs of rats, it exhibits a relaxation effect. Recently, we investigated the second pathway for the relaxation of SMPs. We found that the compound participates in the regulation of enteric neurons expressing nNOS, influences nNOS/NO function and through this mechanism probably regulates the spontaneous CA of gastric SM [28].

These results prompted us to find another method for its synthesis, as well as to obtain more compounds with potential biological activity to investigate ex vivo the influence on SMPs of the compounds compared with papaverine in order to find the pathway of their action.

This relaxation can affect not only the stomach SM but the cerebral vessels as well. Enhanced blood-brain circulation can be one of the reasons for improved cognitive functions. This was the reason for obtaining in vivo pharmacological behavior tests for learning and memory processes in rats.

4.1. In Silico Predictions and Synthesis

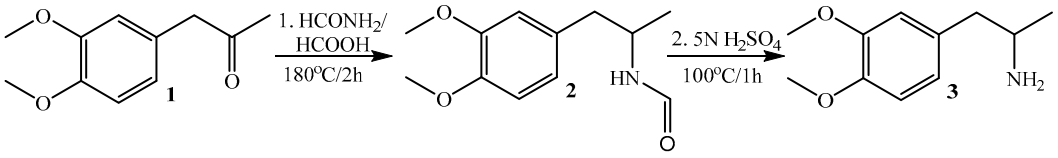
Over the last decade, in silico methods have been applied in the testing processes of new drugs [29]. These in silico simulations consist of computer-based technology, used to predict the biological activities of the compounds [30]. In our calculations the PASSOnline Program was used (Prediction of Activity Spectra for Substances).

In silico simulation predicts muscle relaxant activity for all compounds but only one of them, **4d**, previously mentioned as IQP, showed a relevant effect, so all the biological experiments in the current report are provided for **4d**.

Due to the importance of their synthesis as isoquinoline precursors, some amides were successfully achieved in order to investigate their biological activity and to obtain 1,3-disubstituted isoquinolines in the next step. The amide functional group has great importance in chemistry and pharmacology. Amides form the backbone of peptides, proteins, and other biomolecules [31]. Thus, their synthesis is of crucial importance to the pharmaceutical industry [32].

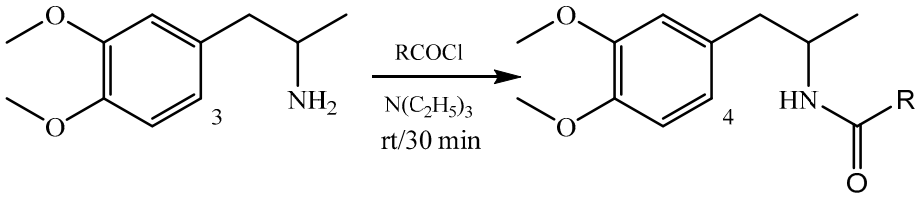
Our strategy was based on the synthesis of 1-(3,4-dimethoxyphenyl)propan-2-amine from starting ketone and its acylation with acyl chlorides to prepare corresponding amides.

To obtain 1-(3,4-dimethoxyphenyl)propan-2-amine, we used a ketone, namely 1-(3,4-dimethoxyphenyl)propan-2-one **1** (commercially available from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), as a starting compound. We found that when applied in Leuckart reaction with HCOOH and $HCONH_2$ and reflux for 2 h at 180 °C it produced N-(1-(3,4-dimethoxyphenyl)propan-2-yl)formamide **2** at first, which after hydrolysis in 5N H_2SO_4 obtained the target product 1-(3,4-dimethoxyphenyl)propan-2-amine **3** with 80% yield according to Scheme 1.



Scheme 1. Synthesis of 1-(3,4-dimethoxyphenyl)propan-2-amine **3**.

The obtained amine **3** was applied in a reaction with different acyl chlorides for the synthesis of amides (Scheme 2).



Scheme 2. Synthesis of N-(1-(3,4-dimethoxyphenyl)propan-2-yl) amides **4**.

In general, reactions with different acyl chlorides proceeded efficiently, furnishing the desired amides **4a**–**e** in 72–91% yield (Table 2). Functional groups, including chlorine, were well tolerated because of the previously found activity of IQP. The resultant compounds are characterized by their melting point, IR, ^{1}H, and ^{13}C-NMR spectra. Spectral data confirmed the structure of all the obtained compounds.

Table 2. Synthesis of N-(1-(3,4-dimethoxyphenyl)propan-2-yl) amides **4**.

4	R	mp, °C	Yield [%]
a	CH_3	83–85	81
b	C_6H_5	103–104	80
c	$CH_2C_6H_5$	120–123	90
d	$CH(Cl)C_6H_5$	87–89	72
e	2-Cl-C_6H_5	88–90	91

In **4d** each starting compound, namely the amine **3** and the acyl chloride α-phenylacetyl chloride, has a stereogenic center, so the final structure possessed two chiral carbons. For that reason, we obtained initially a diastereoisomeric mixture.

We isolated the isomer with the same spectral data as previously reported; 2-chloro-N-((S)-1-(3,4-dimethoxyphenyl)propan-2-yl)-2-phenylacetamide obtained from (S)-alanine [27,28]. That isomer was tested in all biological experiments.

4.2. Ex vivo Experiments on SM Activity

There are two types of SM contractions: phasic and tonic. Phasic SM contraction is characteristic of the gastrointestinal and urogenital systems and displays rhythmic contractile activity. Tonic SM contraction is characteristic of the large arteries and veins and is continuously contracted. SMs with tonic contractile activity are characterized by slow, gradual contraction and relaxation, most often caused by the action of various biologically active substances (drugs, hormones, neurotransmitters, ligands of certain receptors, etc.). Any change in their values provides information about the nature and principle of action of the applied substance.

Biological characterization of the target molecule (IQP) was carried out during our preliminary study using a bioelectrical test [27]. Assuming that the relaxation effect of **4d** is due to its structure (amide and an isoquinoline precursor), we investigated its action independently and in combination with papaverine. Independent exogenous administration of 1×10^{-6} mol/L papaverine or 5×10^{-5} mol/L **4d** causes a relaxation reaction. No significant difference was noticed in the degree and nature of relaxation in the registered SM responses.

Initial administration of **4d** onto SMPs and subsequent exposure to papaverine causes a significantly decreased relaxation reaction. In this experimental model, the partially preserved relaxation of the SMPs is effectuated by the pharmacologically known pathway of papaverine [33,34], which is unaffected by the action of the already administered amide **4d**. When exposed to **4d** following administration of papaverine, the **4d**-induced relaxation reaction disappears completely. In this case, the amide **4d** cannot even partially effectuate its relaxation properties. A possible reason for this is the activation and use of the papaverine-induced pathway of **4d** as an isoquinoline precursor. The same thesis is also confirmed by the results of our experiment in 42×10^{-3} mol/L KCl depolarization. The high content of K^+ in Krebs solution depolarizes the cell membranes [35]. In this environment of already activated voltage-gated calcium channels [36], **4d** does not exhibit its full relaxation effect. All three characteristic parameters (tone, frequency, and amplitude) of spontaneous CA of SMPs [36] from rat stomachs are significantly decreased. The above-mentioned data provide further grounds to suggest an influence on the intracellular Ca^{2+} concentration responsible for the development of SM relaxation after administration of the studied substance. The way **4d** provokes its effects resembles the papaverine-induced reaction on rat stomach SMPs.

We expected that **4d**, as an isoquinoline precursor, would have a cumulative effect on the presence of papaverine. The results did not show exacerbation of the relaxation response caused by either of them independently. We found that when papaverine was applied first, it activated a pathway for relaxation and **4d** could not carry out its potential, thus its effect completely disappeared. We observed a similar effect when **4d** was administered first. Based on these observations we assumed that **4d** uses the same pathway as papaverine.

4.3. In Vivo Methods of Studying Cognitive Functions

Neurodegenerative disorders include a wide range of clinical forms such as Alzheimer's, Parkinson's, and Huntington's diseases, stroke, and amyotrophic lateral sclerosis [37,38]. These brain pathologies are characterized by chronic and progressive neuronal death and degeneration of the function of the central nervus system (CNS), which lead to the decline of different cognitive and motor processes gradually deteriorating the quality of life of patients [39]. Dozens of in vivo studies with variated animal models support the pharmacological potential of an isoquinoline alkaloid berberine for AD; in general this isoquinoline can significantly reduce the cognitive deterioration by modulating multiple apoptotic pathways [40,41]. Several studies have already identified therapeutic effects of papaverine as another isoquinoline alkaloid. Papaverine is a selective inhibitor of PDE10A which is markedly expressed in the striatum of the brain [42,43]; it improved cognitive impairment in a Huntington's disease mouse model by increasing GluA1 and CREB phosphorylation [44].

These facts motivated us to investigate the effect of the synthesized isoquinoline precursors on cognitive functions in rats.

In the shuttle-box active avoidance test with negative reinforcement, the control group showed good results both in terms of acquisition and in the consolidation of memory traces which confirms the validity of the experimental statement. The increased number of conditional stimuli responses (avoidances) is the determining criterion that the task is learned by experimental animals. There is a significant difference in the strength of the pharmacological effect during the training sessions depending on the dose administered. The lowest dose of 5 mg/kg has the most pronounced improving effect on the training process. The significantly increased number of avoidances in the memory test, conducted 7 days after the end of the training, supports the hypothesis that **4d** has an improving effect on long-term memory. Our experiments showed that **4d** in all doses studied has a memory-enhancing effect.

In the step-down passive avoidance test, increased latency of reaction as a criterion for learned tasks confirms the result of the shuttle-box test; significantly prolonged latency of reactions was observed at the lowest dose of **4d** during training.

The significantly increased number of intertrial crossings is a sign of increased motor activity and the stimulating effect of the test compound on the CNS. Only the smallest dose of **4d** showed such effects.

Based on the obtained results, we can assume that 2-chloro-N-(1-(3,4-dimethoxyphenyl) propan-2-yl)-2-phenylacetamide 4d has an improving effect on the cognitive functions of learning and memory.

The synthesis of PDE inhibitors targeting these isoforms of the enzyme, which are localized in the hippocampus and prefrontal cortex, is a promising target for cognitive enhancement.

The compound has an improving effect on cognitive functions of learning and memory in rats. However, it is not dose-dependent; the best effect is observed in the lowest dose. The exact mechanism of action of the compound **4d** has not been established. One possible explanation of the mechanism is the long-term potentiation (LTP) effect. Hippocampal LTP is the most established cellular model for the neuroplastic mechanisms that underlie learning and memory [45]. The few existing studies that investigated the effects of PDE-inhibitors on LTP, did not propose the exact mechanism [7].

5. Conclusions

In conclusion, new amides as isoquinoline precursors were synthesized as a type of bioactive compounds. Preliminary in silico simulations were performed to find any relationship between the structure and its biological activity. This can be detected either ex vivo using functionally active isolated tissues or in vivo. For the ex vivo study of different mechanisms and pathways of action, we used SMs as an appropriate target because of the presence of a wide variety of receptors. We found that one of the compounds, **4d**, caused a relaxation on the SMs using the papaverine pathway. The in vivo experiments showed that the same compound also improved learning and memory processes in rats. The exact mechanism of its pharmacological activity is not clear yet.

Our future plans involve an investigation of MAO and PDE inhibition, as well as ring closure to isoquinoline structure, and a comparative study of the spasmolytic activity and the cognitive function between isoquinolines and their precursors.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm90020037/s1, Figure S1: ^{1}H-NMR spectrum of compound **3**, Figure S2: ^{13}C-NMR spectrum of compound **3**, Figure S3: ^{1}H-NMR spectrum of compound **4a**, Figure S4: ^{13}C-NMR spectrum of compound **4a**, Figure S5: IR spectrum of compound **4a**, Figure S6: ^{1}H-NMR spectrum of compound **4b**, Figure S7: ^{13}C-NMR spectrum of compound **4b**, Figure S8: IR spectrum of compound **4b**, Figure S9: ^{1}H-NMR spectrum of compound **4c**, Figure S10: ^{13}C-NMR spectrum of compound **4c**, Figure S11: IR spectrum of compound **4c**, Figure S12: ^{1}H-NMR spectrum of compound **4d**, Figure S13: ^{13}C-NMR spectrum of compound **4d**, Figure S14: IR spectrum of

compound **4d**, page 15 Figure S15: ^{1}H-NMR spectrum of compound **4e**, Figure S16: ^{13}C-NMR spectrum of compound **4e**, Figure S17: IR spectrum of compound **4e**.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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