UNIVERSITY OF PLOVDIV

"PAISII HILENDARSKI"

Faculty of Chemistry

DEPARTMENT OF ORGANIC CHEMISTRY

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SYNTHESIS OF NOVEL ANTISPASMODICS AFFECTING MEMORY FUNCTIONS IN EXPERIMENTAL ANIMALS

EXTENDED ABSTRACT OF DISSERTATION

for the award of the educational and scientific degree **"DOCTOR" (PHD)**

Field of higher education: 4. Natural Sciences, Mathematics and Informatics

Professional field: 4.2. Chemical Sciences

Doctoral program: Organic Chemistry

Scientific supervisors:

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Plovdiv, 2025

The dissertation is written on 170 standard printed pages and is illustrated with 13 schemes, 12 tables and 32 figures. The bibliographic reference covers 394 literary the sources.

The main part from the doctoral research, including the synthesis and purification of compounds, computer modulation and determination of in vitro anti-inflammatory activity, are carried out in the Faculty of Chemistry of University of Plovdiv „Paisii Hilendarski". The experimental determination on antispasmodic activity, ex vivo anti-inflammatory activity and the influence over memory functions was carried out at the Medical University of Plovdiv. The antimicrobial tests are held at the University of Food Technologies in Plovdiv. MRI analysis is made at the Sofia University "St. Clement Ohridski". HRMS spectra are obtained at the Medical University – Sofia.

The dissertation thesis is discussed on 08.05.2025 by the Extended Department Council of the Department of Organic Chemistry, Faculty of Chemistry, University of Plovdiv „Paisii Hilendarski" and is scheduled for defense before ***a Scientific Jury*** *composed of:*

1. *Prof. Pantelei Petrov Denev, DSc (reviewer)*
2. *Assoc. Prof. Rumyana Ivanova Bakalska, PhD (reviewer)*
3. *Prof. Krastena Todorova Nikolova, PhD*
4. *Prof. Dr. Ginka Atanasova Antova, PhD*
5. *Assoc. Prof. Yordanka Ivanova Uzunova, PhD*

The public defense of the dissertation will take place on 02.07.2025 at 14:00 at Auditorium 1, University of Plovdiv „Paisii Hilendarski".

The materials for the defense are available in the Department of Development of academic composition and doctoral studies and are published on the website of Plovdiv University "Paisiy Hilendarski" – www.uni-plovdiv.bg.

CONTENTS

ABBREVIATIONS USED

SM	Smooth musculature	**ADME**	Absorption, distribution, metabolism and excretion
SMP	Smooth muscle preparations	**ASA**	Acetylsalicylic acid
IR (FT-IR)	Infrared	**BBB**	Blood-brain barrier
NSAIDs	Non-steroidal anti-inflammatory drugs	**CCh**	Carbachol
SCA	Spontaneous contractile activity	**IBD**	Inflammatory bowel disease
GIT	Gastrointestinal tract	**IBS**	Irritable bowel syndrom
SAIDs	Steroidal anti-inflammatory drugs	**IL**	Interleukin
CNS	Central nervous system	**LD50**	Lethal dose, 50%
NMR (NMR)	Nuclear magnetic resonance imaging	**nNOS**	Neurological nitrogenous oxide synthase
5-HT	5-hydroxy tryptamine, serotonin	**NO**	Nitrogen oxide
ACh	Acetylcholine	**TPSA**	Topological polar superface area

INTRODUCTION

Functional and inflammatory disorders of the gastrointestinal tract, such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), remain a serious clinical challenge with increasing social and medical importance. They are characterized by a complex and still poorly understood pathophysiology, in which various factors are involved - intestinal motility disorders, visceral innervation hypersensitivity, immune response disorders, impaired intestinal barrier and altered microbiota. As a result of this complexity, available therapeutic options are often symptomatic, with limited efficacy and undesirable side effects in long-term use.

In current therapeutic practice, antispasmodics occupy a central place in the treatment of IBS and other functional gastrointestinal disorders. Their effect is based on smooth muscle (SM) relaxation, calcium channel modulation and anticholinergic activity. Despite their established use, existing molecules often do not achieve the desired clinical outcome, especially in patients with severe or refractory forms of the disease, and can cause adverse reactions such as dry mouth, dizziness or cardiac arrhythmias. This highlights the need to discover new drug molecules with an improved pharmacological profile – higher efficacy, lower toxicity and a broader spectrum of action.

One of the modern strategies in drug design is the development of **hybrid molecules,** combining pharmacophoric elements with different mechanisms of action within a single chemical structure. This approach allows for simultaneous targeting of different links in the pathophysiological cascade, potentially leading to a synergistic therapeutic effect and reduction of the required dose and, accordingly, of unwanted effects. As a structural basis for the creation of such hybrids, **anthranilic acid** represents a particularly interesting object – it is well studied, present in a number of bioactive compounds with anti-inflammatory, analgesic, antiallergic and antispasmodic effects and offers numerous possibilities for chemical modification.

The study of the biological activity of these newly synthesized hybrid structures is based on a combination of modern approaches – ***in silico*** **modeling** to predict the biological profile, **chemical synthesis** of selected molecules and **experimental biological testing.** *Ex vivo* models with isolated sM are used to assess the contractile activity and clarify the mechanism of action, while *in vitro* tests reveal additional aspects such as anti-inflammatory and antimicrobial potential. The conducted *in vivo* studies in experimental animals allow to track systemic effects, including the influence on cognitive parameters – a factor of increasing importance in chronic diseases associated with stress and altered quality of life. The obtained results are compared with established reference agents such as mebeverine, diclofenac, acetylsalicylic acid and prednisolone.

The present dissertation presents original results from the synthesis, characterization and pharmacological evaluation of novel hybrid derivatives of anthranilic acid as potential antispasmodics with additional anti-inflammatory and cognitive-modulating effects. The developed structures contribute to expanding the set of available structural analogues, deepen the understanding of the structure-activity relationship and reveal new possibilities for the therapy of complex conditions such as IBS and IBD.

PURPOSE AND OBJECTIVES OF THE DISSERTATION

Key priorities for pharmaceutical chemistry remain the discovery of new therapeutic strategies, the optimization of established pharmacological agents to achieve better binding to target receptors, or the validation of new agents with a different mechanism of action. basis on pharmacological activities on derivatives on anthranilic acid, is formulated the goal on the dissertation labor: **synthesis on new derivatives on anthranilic acid acid as new candidates for antispasmodics and substances affecting memory functions at experimental animals.**

To achieve the research goal, the following tasks have been set:

1. ***In silico* assessment of the biological activity and potential toxicity** of new hybrid molecules.
2. **Synthesis of a starting hybrid molecule** combining pharmacophore residues of anthranilic acid and substituted 2-phenylethylamines.
3. **Preparation of amides of the hybrid molecule** in order to create derivatives with improved antispasmodic and anti-inflammatory activity.
4. **Purification and characterization** of all synthesized compounds with appropriate analytical methods.
5. **Determination of antispasmodic and anti-inflammatory activity** of the obtained compounds in comparison with used antispasmodics such as mebeverine and anti-inflammatory agents such as diclofenac, acetylsalicylic acid and prednisolone.
6. **Evaluation of the antimicrobial activity** of the synthesized compounds.
7. **Determining the effect of substances on memory functions** in experimental animals.

MATERIALS AND METHODS

Materials:

All used reagents and solvents are purchased from Merck.

The medicinal products used for comparison were purchased from local pharmacies.

Method for synthesis of output hybrid molecules:

Mixture of isatoic anhydride (1.63 g, 10 mmol) and the corresponding 2-phenylethylamine (2.10 mL, 15 mmol) in dichloromethane (30 mL) were stirred at room temperature for 24 hours.

The obtained solution is filtered through neutral Al_2O_3 and is concentrated.

Method for synthesis of diamide derivatives:

To a solution of 3 mmol of the corresponding hybrid **3**, **4** or **5** in 10 mL of dichloromethane, 3.5 mmol of the corresponding acid chloride were added.

$N(C_2H_5)_3$ was added and after another 30 minutes, the reaction mixture was washed sequentially with dilute HCl (1:4), Na_2CO_3, and H_2O.

After drying with anhydrous Na_2SO_4, the solution was filtered on a column of neutral Al_2O_3 and concentrated.

Chromatographic, spectral and computer methods:

→ Thin layer chromatography: used chromatographic plates Fluka 0.2 mm silica gel and chromatographic solvent system chloroform: diethyl ether: n-hexane = 6:3:1.

→ Preparative column chromatography: neutral Al_2O_3 is used for filtration and separation.

→ ^{1}H-NMR and ^{13}C-NMR spectra are recorded on Bruker Avance III HD 500 spectrometer (Bruker) at 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR) and room temperature (295 K). The chemical offsets are expressed in parts per million (ppm), carried away to tetramethylsilane (TMS) (δ = 0.00 ppm) as internal standard. The constants on spin-spin interaction are specified in Hz.

→ Infrared spectra were recorded with a VERTEX 70 FT-IR spectrometer (Bruker Optics) and peak shifts are indicated in cm $^{-1}$.

→ Melting points were determined with a Kruss M5000 apparatus (A. Krüss Optronic GmbH) and are presented in degrees Celsius (°C).

→ HRESIMS spectra were obtained in positive mode on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific, Inc.) with HESI-II.

→ The following freely available software products were used for screening of biological effects, pharmacokinetic properties and toxicity: PASS Online, SwissADME, ProTox - II, OSIRIS20.

→ Statistical analysis of experimental data with Instat and IBM SPSS Statistics v.26.

Methods for assessing the biological activity of compounds:

→ Isometric determination of *ex vivo* antispasmodic activity on rat gastric smooth muscle preparations in a tissue bath upon incubation with hybrid compounds, neurotransmitters and known antispasmodics.

→ Determination of *in vitro* inhibition of thermally induced denaturation of human serum albumin.

→ *Ex vivo* immunohistochemical determination of IL-1β and nNOS expression in smooth muscle and myenteric plexus tissue preparations of Wister rat stomach.

→ Determination of *in vitro* antimicrobial activity using the agar diffusion method.

→ *In vivo* study of the influence of newly synthesized hybrid compounds on rat locomotor activity and cognitive functions.

The experimental animals (Wister rats) were raised in the "Vivarium" of the Medical University of Plovdiv – Plovdiv. The internationally established principles for working with experimental animals, regulated by EU Directive 86/609/EEC, were strictly observed. The experiments were performed in strict compliance with the requirements of the Bulgarian Food Safety Agency (Permit for working with experimental animals No. 229, valid until 11.04.2024 and No. 400, valid until 06.06.2029).

RESULTS AND DISCUSSION

Polypharmacology is not a new concept, but there are different approaches to the simultaneous administration of two or more drugs. The most commonly used strategies include: simultaneous administration of several individual drugs, combining several active substances in a single dosage form, as well as the creation of hybrid molecular structures capable of interacting with multiple therapeutic targets. Due to the increased risk of toxicity in combination chemotherapy, increasing attention is being paid to the development of single hybrid molecules combining different pharmacophore fragments (multi-ligand approach). In recent decades, intensive progress has been observed in the synthesis of such hybrids, including biologically relevant structural elements, which are being investigated as potential pharmacologically active substances and drugs.

The therapeutic efficacy of a drug is determined by a number of physicochemical characteristics, including absorption, distribution, metabolism, interaction with target molecules, excretion, and toxicity. Optimization of these parameters requires targeted molecular design. Due to the high value of structural modification of already known pharmacophores, hybrid compounds formed by combining structural and functional features of two different biologically active fragments represent a preferred class of molecules for the creation of new drug candidates with improved properties.

Drawing from a wide variety of natural and synthetic molecular structures with proven biological activity, these hybrid compounds often demonstrate synergistic or emerging pharmacological properties.

1. IN *SILICO* SCREENING

There are many factors that limit the systematic use of experiments in the pre-clinical stage of new medicines discovery. Among these factors are the multitude of newly synthesized molecules, quantitative restrictions on tissue samples, as well as limitation on animal experiments. In this context, it is reasonable to consider supplementing or partially replace biological tests with computer models. Concerning that, we decided to implemetn software products in the present study, in order to pre-screen a theoretical library of new hybrid molecules as potential candidates for medicines. To be successfully used as a medicine, one molecule must reach its pharmacological target in the body in a suitable concentration and to remain in bioactive form long enough. A lot of promising biologically active substances fail to succeed as medicines because of their low bioavailability and poor pharmacokinetic properties (Figure 1).

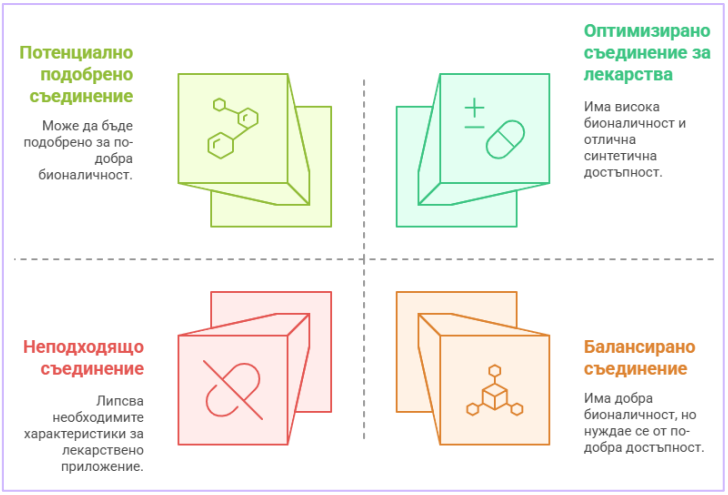


Figure 1. Assessment of the bioavailability profile of compounds.

In relation to the aim of the dissertation, we created a targeted pharmacophore model that would inhibit the action of the endogenous neurotransmitter acetylcholine (ACh). To select target structures to be synthesized, we used the network pharmacology method described by Xiao, and applied the following databases: TTD (http://bidd. nus.edu.sg/group/cjttd/), DrugBank (https://www.drugbank.ca/), GeneCards (https://www.genecards.org/), DisGeNET (https://www. disgenet.org/), and OMIM (https://omim.org/). To find target proteins related to gastrointestinal spasms, we used a database search with the keywords“Spasm”;“Spasms”;“Muscular spasm”,“Muscle spasms”. The key target genes and compounds were then identified in a general diagram that focused on the synthesis of compounds affecting the calcium signaling pathway. The literature review showed that anthranilic acid derivatives have similar effects and are modulators of various ion channels, including L-type calcium channels. **Therefore, as the first pharmacophore unit, we chose anthranilic acid derivatives to be the main structural element included in the synthesis of hybrid molecules as potential drug candidates against inflammatory diseases, including gastrointestinal ones.**

When studying the effects of a compound on calcium channels, it is important to consider the influence of phenylethylamine calcium channel blockers. **In this context, the**

structure of 2-phenylethylamine was chosen as the second pharmacophore unit in the design of the hybrid molecule. Phenylethylamines represent a large class of compounds of biogenic or synthetic origin. Amphetamine is a represenative of the synthetic subgroup, familiar with its stimulating effects. The biogenic subgroup consists of well-characterized neurotransmitters, such as dopamine, norepinephrine, serotonin, and β- phenylethylamine. In the brain of mammals β-phenylethylamine is unevenly distributed as highest concentrations is observed in the limbic structures. These areas are strongly innervated from dopaminergic neurons and show high sensitivity to amphetamine. Changes in metabolism of β-phenylethylamines are open at neurological disorders, including schizophrenia and hyperactivity deficit disorder on the attention that suggests the participation of this amine in pathophysiology on monoaminergic systems. Phenethylamines, tryptamines and ergolines are the three categories in which often fall the structures of 5-HT_{2A} receptors agonists, but the most attention has been drawn to the class of phenylethylamines as selective agonist of 5-HT subtype receptors (Figure 2). The neurotransmitter serotonin (5-HT) plays key role for the mood, libido, aggression, anxiety, cognition, sleep, appetite, pain, and nutrition, as well as peripheral circulatory, gastrointestinal, endocrine and respiratory systems regulation.

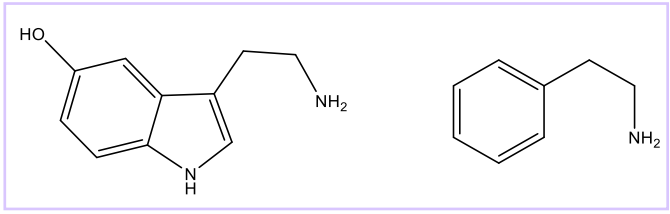
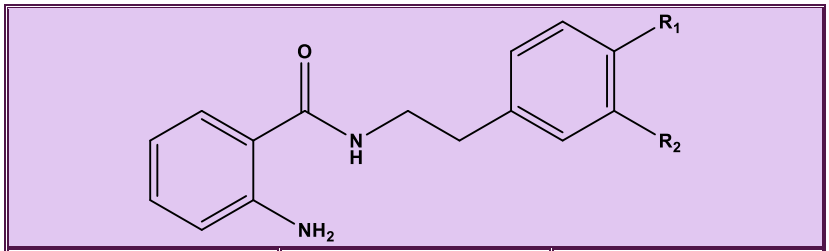


Figure 2. Chemical structures of 5-hydroxytryptamine (5-HT) and phenylethylamine.

When chosing derivatives of the 2-phenylethylamines, we directed our attention to three types: unsubstituted 2-phenylethylamine, 3-chloro-2-phenylethylamine and 3,4-dimethoxyphenylethylamine (Figure 3). The goal was to explore the influence on the substitutes on antispasmodic and anti-inflammatory activity, with a view to selection on the most suitable compound for subsequent *in vivo* research.

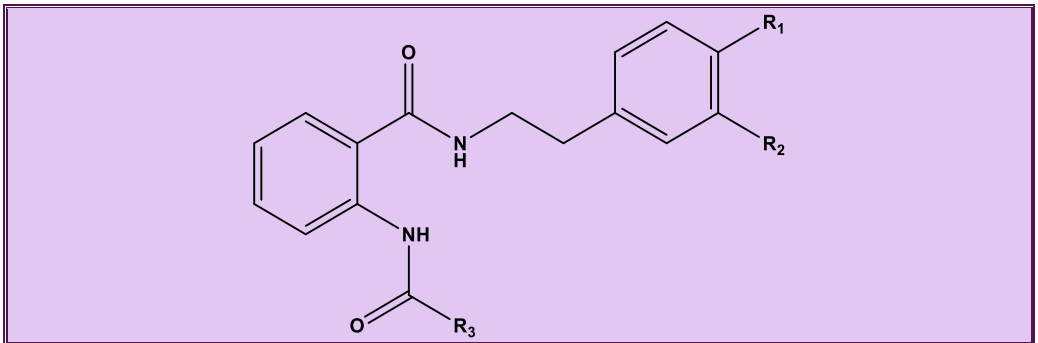


$R_1 = R_2 = H$	R_1 =Cl, R_2 = H	$R_1 = R_2 = OCH_3$
3	**4**	**5**

Figure 3. Selected 2-phenylethylamine derivatives for the synthesis of hybrid molecules.

Planning to obtain a larger number of new biologically active derivatives, the next focus was the selection of anthranilic acid diamides for synthesis. The amide bond is ofcrucial importance in medicinal chemistry, and very frequently discovered in biologically active molecules and its wide application at synthesis on peptides, proteins and others macromolecules. The amide bond is characteristic for many therapeutic agents and is found in

approximately one quarter of all approved drug products. The ability of amides to interact with biological targets must also be taken into account when designing new drugs. In this context, as well as in order to study the influence of different substituents on the structure and properties, the synthesis of derivatives containing alkyl and aryl residues is of interest in the present work. As alkyl substituents, methyl one was chosen, and from the aryl class – phenyl, 2-chlorophenyl, benzyl and α-chlorobenzyl (Figure 4).



$R_1 = R_2 = H$	R_1 =Cl, R_2 = H	$R_1 = R_2 = OCH_3$	R_3
6a	7a	8a	CH_3
6b	7b	8b	C_6H_5
6c	7c	8c	$CH_2C_6H_5$
6d	7d	8d	2-Cl-C_6H_4
6e	7e	8e	CH(Cl)-C_6H_5

Figure 4. Selected alkyl and aryl substituents for the synthesis of diamides.

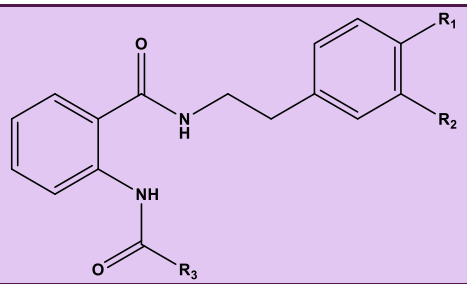
Initially we conducted *in silico* tests for each one from the compounds, in order to determinable their pharmacokinetic properties. The properties on absorption, distribution, metabolism and excretion (ADME) of targeted compounds in the presented dissertation are rated in *silico* using on web tools SwissADME, OSIRIS20 and ProTox -II. These programs give common values for assessment of the compounds, in order to significantly reduction on the unsuccessful clinical tests. In our research were rated molecular descriptors as coefficient on distribution (Log Po/w), molecular weight, donors and acceptors on hydrogen connections, topological polar superficial area (TPSA), violations on the rule on Lipinski et al. The expected biological effects were predicted with the PASS (Prediction of Activity Spectra for Substances) program.

In the analysis of the target hybrid compounds, we applied the Lipinski rule – widely used in drug discovery to assess the properties upon oral administration. It defines some key criteria for drug similarity. According to the rule for candidates for medicines must be fulfilled at least two of the four basic pharmacokinetic properties (MW ≤ 500, XLOGP3 ≤ 5, number donors on hydrogen bonds ≤ 5 and number of acceptors on hydrogen bonds ≤ 10,6); lipophilicity (XLOGP3: – 0,7 to +5,0), molecular weight (MW: 150 to 500 g/mol), polarity (TPSA: 20–130 Å2), ESOL or evaluated solubility (log S: no more of 6), saturation (Fraction Csp ^{3} or faction on carbon atoms in sp^3 hybridization: no less of 0.25) and number of conformationally free bonds (RB: less than 9).

Lipophilicity is a key characteristic that plays main role at the assessment on bioavailability. According to computer modeling, the values of log P are lower out of 5, which

suggests good bioavailability (Table 1). The study of ADME shows that the compounds pass through the blood-brain barrier barrier (BBB) and have high gastrointestinal absorption and can be considered as a drug candidate. TPSA is a descriptor that provides information for transport properties on the medicine, as intestinal absorption (TPSA below 140 Å2) and penetration through BBB. The calculated values of TPSA for compounds **3–5** are in the range 55.12–73.58 Å2, which respond on the criteria for effectively intestinal absorption and crossing the BBB. The number on conformationally free connections for all compounds is lower from nine, which corresponds on sufficient oral bioavailability. Solubility is another important property that influences on absorption. Compounds are expected to be moderate soluble in water according to the parameter log S. The estimate through radar model for bioavailability also confirmed that all planned for synthesis compounds respond on the criteria for medicinal purposes similarity, except on number of carbons atoms in sp^3-hybridization.

Table 1. Pharmacokinetic properties of hybrid molecules 3 – 5 and their diamides obtained after in silico analysis.



Compound	R3	MW g/mol	log S ESOL	Log P o/w	Log Kp cm/s	TPSA Å 2	H-acceptor	H-donor	RB	C sp^3	GIA
3	–	240.30	−3.08	2.46	−5.98	55.12	1	2	5	0.13	high
4	–	274.75	−3.98	3.06	−5.38	55.12	1	2	5	0.13	high
5	–	300.35	−3.51	2.56	−6.02	73.58	3	2	7	0.24	high
6a	CH_3	282.34	−3.81	2.86	−5.47	58.20	2	2	7	0.18	High
7a	CH_3	316.78	−3.96	3.23	−5.74	58.20	2	2	7	0.18	High
8a	CH_3	342.39	−3.51	2.59	−6.38	76.66	4	2	9	0.26	High
6b	C_6H_5	344.41	−4.82	3.89	−5.18	58.20	2	2	8	0.09	High
7 b	C_6H_5	378.85	−5.41	4.34	−4.94	58.20	2	2	8	0.09	High
8b	C_6H_5	404.46	−4.95	3.82	3.82	76.66	4	2	10	0.17	High
6c	$CH_2C_6H_5$	358.43	–4.78	3.92	–5.31	58.20	2	2	9	0.13	High
7c	$CH_2C_6H_5$	392.88	–5.38	4.40	–5.07	58.20	2	2	9	0.13	High
8c	$CH_2C_6H_5$	418.48	–4.92	3.86	–5.71	76.66	4	2	11	0.20	High
6d	2-Cl-C_6H_4	378.85	–5.41	4.33	–4.94	58.20	2	2	8	0.09	High
7d	2-Cl-C_6H_4	413.30	–6.00	4.88	–4.70	58.20	2	2	8	0.09	High
8d	2-Cl-C_6H_4	438.90	–5.55	4.25	–5.35	76.66	4	2	10	0.17	High
6e	$CH(Cl)C_6H_5$	392.88	−5.43	4.31	−5.00	58.20	2	2	9	0.13	High
7e	$CH(Cl)C_6H_5$	427.32	−6.03	4.67	−4.77	58.20	2	2	9	0.13	High
8e	$CH(Cl)C_6H_5$	452.93	−5.58	4.25	−5.91	76.66	4	2	11	0.20	High

Legend: *Compounds **6a–e** – $R_1 = R_2 = H$; Compounds **7a–e** – $R_1 = Cl$, $R_2 = H$; Compounds **8a–e** – $R_1 = R_2 = OCH_3$. MW: molecular weight; Log P o/w: coefficient on distribution octanol / water; TPSA: topological polar area; ESOL LogS: calculaed solubility in water; Fraction Csp 3: ratio on sp^3 hybridized carbon atoms compared to the general number carbons; RB: flexibility (number of conformationally free bonds); H-acceptor – number of hydrogen bond acceptors; H-donor – number of hydrogen bond donors, GIA – gastrointestinal absorption.*

The *in silico* toxicity assessment (ProTox–II) for most from the compounds gives approximately 70% probability for respiratory toxicity, as well as potential for toxic effects on CNS after passage through the BBB. Similar undesirable reactions often are observed at active substances intended for treatment of complex neurodegenerative diseases. According to the pre-calculated data for acute toxicity, the three hybrid compounds belong to class 4 (LD_{50} = 1000 mg/kg), and their diamide derivatives range between 1000–2025 mg/kg.

The computer modeling confirmed validity on the hypothesis that each one of the compounds 3–8 can is accept as potential therapeutic candidate. This gave us the reason to synthesize the indicated compounds in order to obtain a "small library" of hybrid molecules and determine their antispasmodic and anti-inflammatory activity, as well as identify suitable candidates for in vivo cognitive tests on experimental animals.

2. SYNTHESIS AND CHARACTERIZATION OF TARGET COMPOUNDS

There are several methods for the synthesis of anthranilic acid derivatives. The most common one involves the following steps:

→ acylation with acylating agent agent on aniline or substituted aniline at suitable conditions on temperature and pressure for formation on the relevant one acylated product;

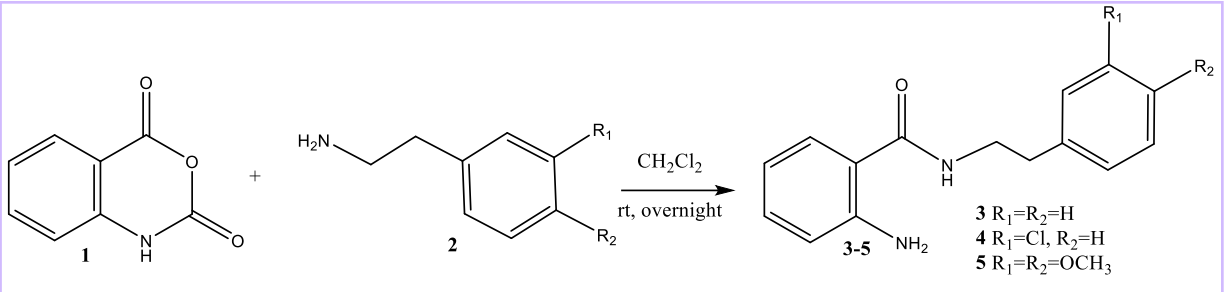
→ subjection on acylated product in conditions on halogenation in the presence on oxidizer;

→ subjection on the product in conditions on carbonylation at suitable temperature and pressure, for is forms anthranilic acid, from which in the next step can is receive amide and ester derivatives.

The Chosen One synthetic approach in this dissertation work founded on other method for synthesis, namely opening on the ring on Izatoev anhydride at interaction with 2-phenylethylamines with modifications from descriptions in the literature

2.1. Synthesis of hybrid molecules

A reaction was carried out with 2-phenylethylamine, 2-(3-chlorophenyl)ethylamine and homoveratrylamine in dichloromethane. The course of the reaction was monitored by thin layer chromatography, and the target products were isolated in practically quantitative yields (Scheme 1).



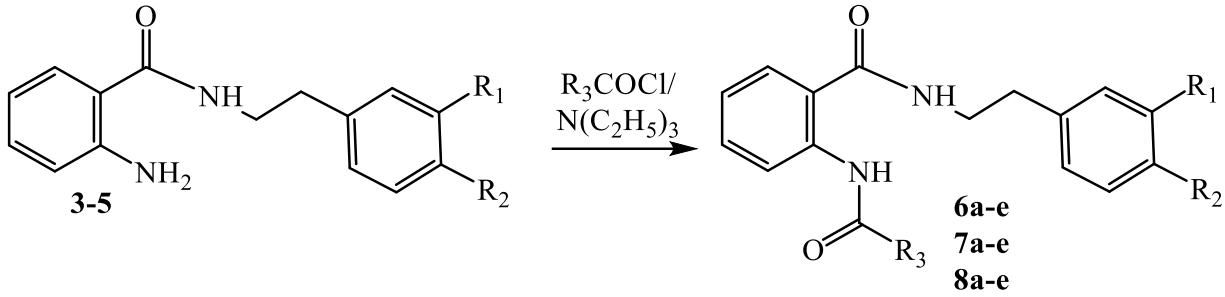
Scheme 1. Synthesis of amides 3–5.

The structure on received compounds is confirmed with the help of of FT-IR, NMR, HRMS- spectral data. In the FT- IR spectra are observed stripes for amino group, amide group and substituted benzene core. In the ^{1}H-NMR spectra and the ^{13}C-NMR spectra all signals were completely compatible with the expected structures of the hybrid compounds **3-5.** In ^{1} H- NMR observe wide singlets at 5.5 ppm for NH_2 group and 6.6 ppm for NH from the amide group in **4;** 5.5 ppm for NH_2 group and 6.2 ppm for NH from the amide group in **5;** and 5.5 ppm for NH 2 group and 6.1 ppm for NH from the amide group at compound **3.** Except this in everyone one from the spectra is observe the characteristic for the triplet and quartet 2-phenylethylamines for for CH_2 groups and signals for aromatic ring from 2-phenylethylamine and from anthranilic acid acid. In ^{13} C-NMR of **4** is appears signal for the C=O group at 169.4 ppm, as well as signals for aromatic ring and CH_2 groups at 40.6 and 35.5 ppm. The FT-IR spectrum also shows bands for NH, NH_2 groups, amide group and metasubstituted benzene nucleus. In ^{13} C-NMR of the compounds is appears signal for the C=O group at 169.2 ppm, as well as signals for aromatic ring and CH_2 groups in the region 40.0–41.0 and 35.2–35.5 ppm. The molecular table on compounds was confirmed by HRMS.

2.2. Synthesis of diamides of hybrid molecules

In order to receiving on new potentially biologically active derivatives, the next stage in synthesis a is the receipt on diamides on received hybrid molecules **3**–**5.** For its implementation we applied newly synthesized hybrid and in reactions and of acylation with a set of five acyl chlorides (Scheme 2).

The reaction with acidic chlorides is effective and provides the desired diamides **6 a–e, 7 a–e** and **8 a–e** with yields between 78–83 % (Table 2). The functional groups, including phenyl and benzylic substitutes, were preferred because of the previously conducted *in silico* screening.



Scheme 2. Synthesis of diamides 6a–e, 7a–e, 8a–e.

Table 2. Synthesis of anthranilic acid diamides – yields and melting point temperatures (MP).

Compound	R_1	R_2	R_3	Yield, %	MP, °C
6a	H	H	CH_3	78	90–92
6b	H	H	C_6H_5	80	94–95
6c	H	H	CH_2-C_6H_5	81	85–86
6d	H	H	2-Cl-C_6H_4	80	161–164
6e	H	H	$CH(Cl)C_6H_5$	79	76–77
7a	Cl	H	CH_3	83	135–137
7b	Cl	H	C_6H_5	79	106–108
7c	Cl	H	CH_2-C_6H_5	79	82–83
7d	Cl	H	2-Cl-C_6H_4	80	61–63
7e	Cl	H	$CH(Cl)C_6H_5$	81	83–84
8a	OCH_3	OCH_3	CH_3	80	95–97
8b	OCH_3	OCH_3	C_6H_5	78	121–124
8c	OCH_3	OCH_3	CH_2-C_6H_5	79	92–93
8d	OCH_3	OCH_3	2-Cl-C_6H_4	81	94–95
8e	OCH_3	OCH_3	$CH(Cl)C_6H_5$	82	102–103

The obtained compounds were characterized with their melting point temperatures, ^{1}H, ^{1} C- NMR and HRESIMS spectra. The spectral data confirm the structure of all synthesized compounds. In ^{1}H-NMR a broad singlet appears in the region 10.5 – 12.0 ppm for a proton from the new amide group, as well as a signal for the new carbonyl group on the second carbonyl carbon atom in ^{13} C-NMR. In the carbon spectrum of the compound the appearance of a new carbonyl group at 168.9 ppm is observed.

3. BIOLOGICAL ACTIVITY OF THE RESULTING HYBRID COMPOUNDS

3.1. *Ex vivo* effect of hybrid molecules on contractile activity of smooth muscles

The assessment on pharmacological profile on all 18 hybrid compounds was accomplished through *ex vivo* experiments on isolated fabrics from the corps part on stomach on rat. Researched were changes in amplitude, frequency and tonic component on spontaneous contractile reactions compared to those found after administration on the reference antispasmodic mebeverine, the neurotransmitters acetylcholine (ACh) and carbachol (CCh), through strain gauge registration.

The action on newly synthesized molecules **3** and **6a–e** was compared to these on mebeverine at submaximal concentration (5.10^{-5} mol/L) versus concentration range $10^{-6} \div 10^{-4}$ mol/L. Compound **6e** no caused essential change in contractility activity. Similar on mebeverine action was established for **6c.** Most pronounced relaxation effect showed the diamide derivatives **6b** and **6d** (Figure 5). Essential difference is register between mebeverine and the studied compounds at tracking on the influence them on ACh-mediated SM contractions. Neither one from the newcompounds (**3, 6a–e)** no affects significant ACh-response (changes is registered in the range 0.5% ÷ 1.4%), for difference from mebeverine, which caused 99.3% inhibition on the answer at the same concentration. These results are an indication of an adverse effect caused from mebeverine through blocking on ACh – important neurotransmitter in neuromuscular communication. This raises questions regarding safety of the antispasmodic drug at long-term use and impose search on new compounds with similar relaxing effect, but with a better profile on safety.

In response to this challenge through medicinal design and molecular modeling have been identified two compounds from the first group of hybrids with unsubstituted 2-phenylethylamine (6b and 6d), which keep the purposeful relaxing action on SM, but manifest less unwanted effects. We think that this is owes on the presence on phenyl remainder in their structure (6b, $R_3 = C_6H_5$; 6d, R_3 = 2-Cl-C_6H_4).

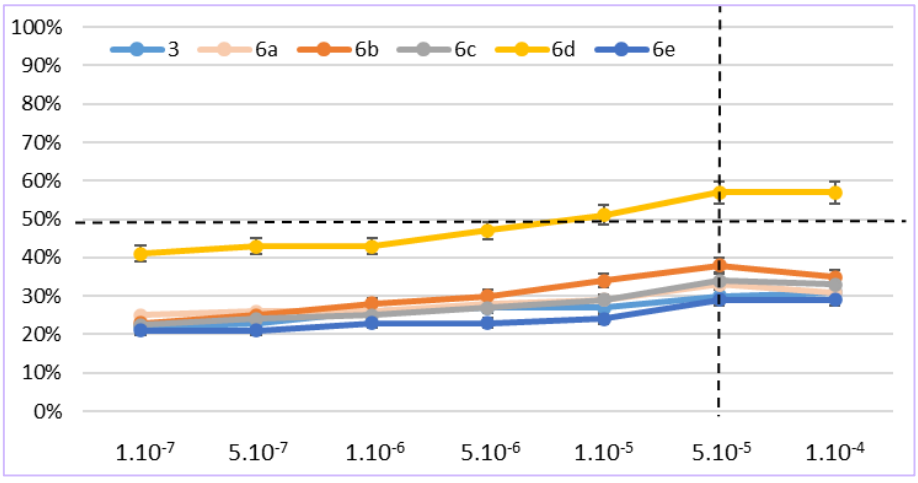


Figure 5. Changes (%) in the abbreviation activity of SMP, induced of 3 and 6a–e in the concentration range range from 1.10^{-7} to 1.10^{-4} mol/L (number of repetitions n = 8).

The relaxing one action on in advance predicted *in silico* compounds in group (**4, 7a–7e**), selected by difference in amide group related to anthranilic acid, was appreciated through their application on CCh-precontracted smooth musculature. The effects are expressed in percentage reduction on the induced from CCh tonic contraction (Figure 6). The statistical analysis on spontaneous contractile activity (SCA) showed that all investigated compounds suppress the spontaneous smooth muscle activity in dependence from concentration (0–72%), as confirm the potential you as relaxants-spasmolytics.

Compound 4 demonstrates most pronounced relaxation (59% at 5.10^{-5} mol/L), surpassing 7a–7e. Its hybrid structure provides higher efficacy compared to other analogues, which makes it a leading candidate for the development of a new therapeutic agent for conditions associated with increased muscle tone.

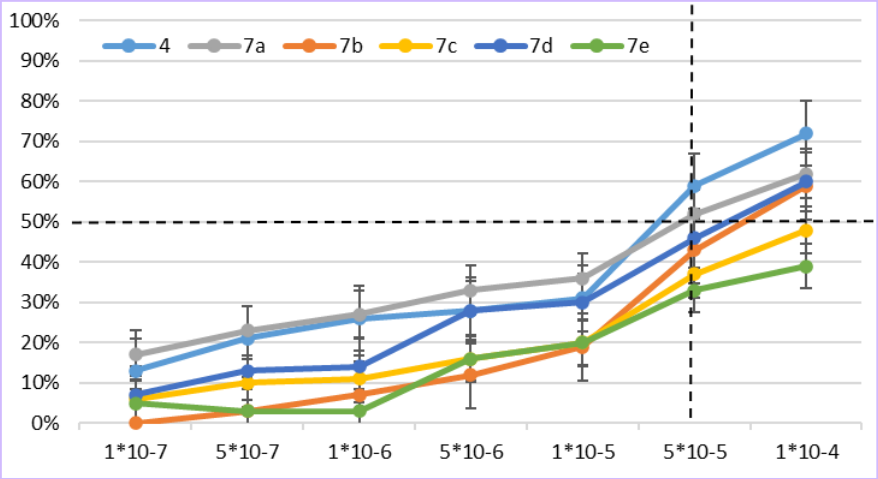


Figure 6. Relaxation responses induced by compounds 4 and 7a–e in the concentration range of 1.10^{-7} to 1.10^{-4} mol/L, on SMPs precontracted with CCh. The effect of 10^{-7} mol/L CCh was taken as 100% and all subsequent responses are expressed as a percentage of this value.

At identical conditions on incubation on the isolated tissues in the organ bath, were investigated the influences and chemical compounds from the last group **of** hybrids with 3,4-dimethoxy-2-phenylethylamine: **5** (n = 7), **8a** (n = 8), **8b** (n = 8), **8c** (n = 7), **8d** (n = 7) and **8e** (n = 8). The most strongly pronounced tonic relaxation effect was registered at concentration 5.10^{-5} mol/L for three from hybrid molecules from this one group substances, respectively: **8c** —40%, **8d** —54% and **8e** —44% (Figure 7).

From the specified three substances containing 2-chlorophenyl, benzyl and alpha-chlorobenzyl substituent, we found that the most active tonic relaxants are 8d and 8e.

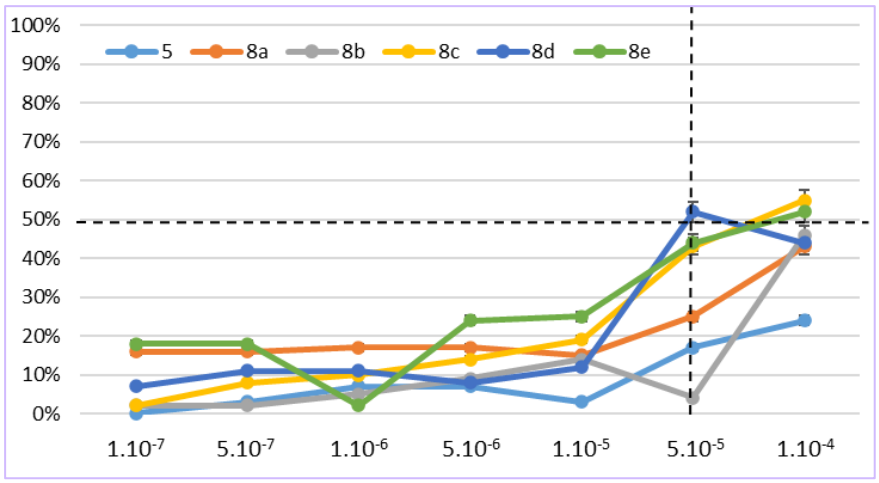


Figure 7. Relaxation responses induced by compounds 5 and 8a–e in the concentration range of 1.10 $^{-7}$ to 1.10 $^{-4}$ mol/L, on SMPs precontracted with CCh. The effect of 10 $^{-7}$ mol/L CCh was taken as 100% and all subsequent responses are expressed as a percentage of this value.

SMPs, in which they are most strongly observed the expressed five relaxation the effect (caused by the compounds **6b, 6d, 4, 8d** and **8f)**, showed gradually and steadily relaxation on the muscles – typically for tonic and SM relaxation, associated with slow regulation on calcium homeostasis. In the analysis on the results by groups, distinguishing is by the 2-phenylethylamine residue and by acyl component, we found that the action of on these hybrids them determines as potentially useful at the treatment on conditions related with cramps and increased muscular tone, including multifactorial intestinal diseases and syndromes (IBD and IBS). Differences in activity on interaction with biological targets and in the degree on influence on the different components on spontaneous abbreviations obviously is due to the different chemical structure. This data they assume that structural variations in the molecule can play key role in modification on pharmacological properties on the new therapeutic agents as selective agonists or antagonists on SM.

For full characteristic on all 18 synthesized muscle relaxants, we also assessed the potential them for amplitude and/ or frequency relaxation – faster and shorter-lasting effect, result from abruptly decrease in the concentration of intracellular Ca^{2+}, leading to suppression on the rhythmic contractions. Grouping was by type on the substituent: H (A); CH_3 (B); C_6H_5 (C); $CH_2C_6H_5$ (D); 2-Cl-C_6H_4 (E); CH(Cl)-C_6H_5 (F) in the structure on molecules (Figure 8).

We have established that the frequency on contractions of SMP is identical to a by strength and character and remains almost unchanged, varying in many narrow interval between 96% and 100% for all investigated compounds, applied at a concentration of 5.10 $^{-5}$ mol/L. This is statistically insignificant difference and indicates minimal to missing frequency effect (Figure 8). This experimental result shows that the researched hybrid molecules they don't have essential effect on the frequency on the phasic contractions of SM at the indicated concentration and no influence structures and intracellular processes responsible from his/her own country for generation and regulation on the frequency on spontaneous contractions in the SM.

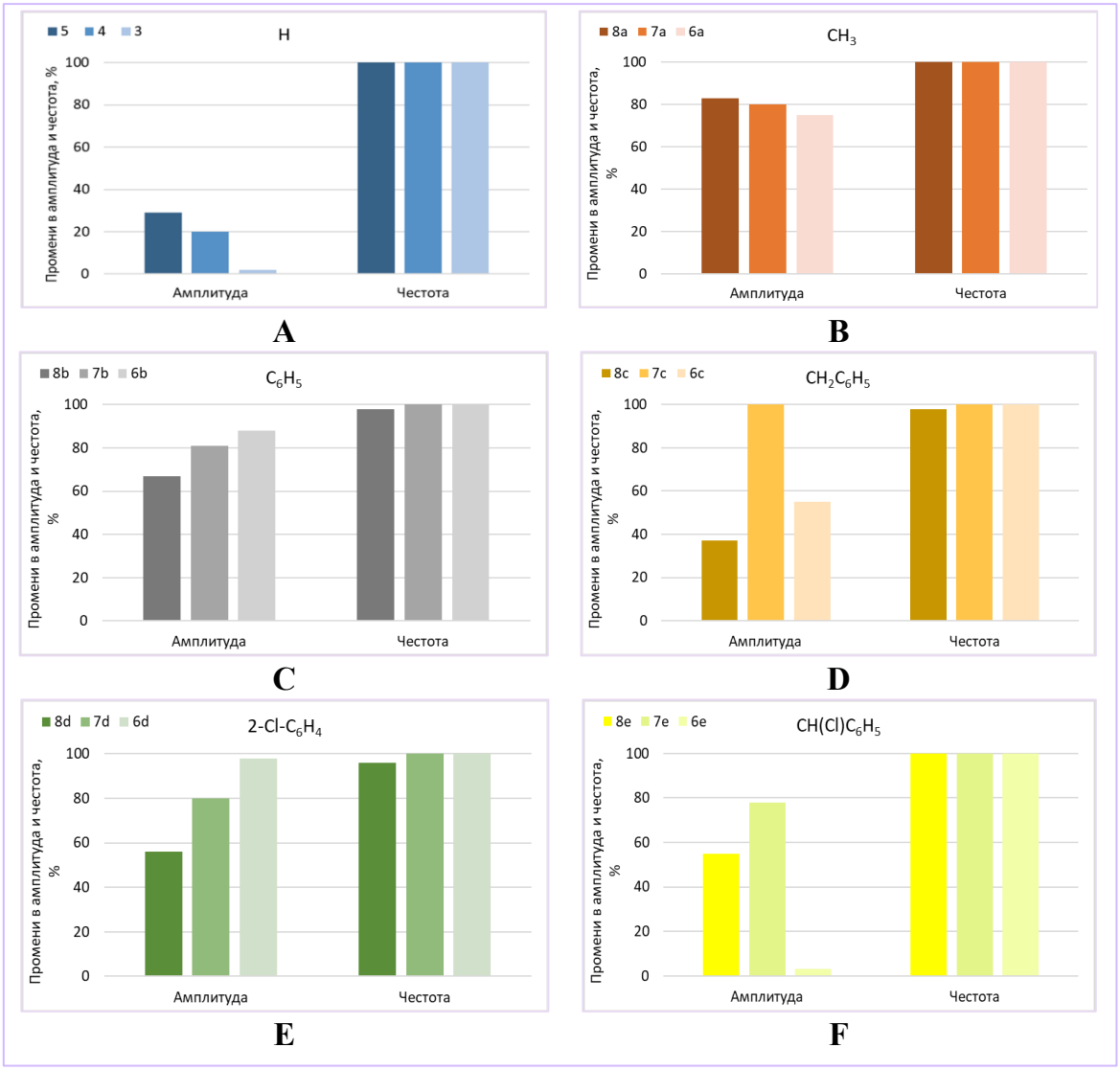


Figure 8. Change in amplitude and frequency of spontaneous SMP contractions induced by compounds 3, 6a–e, 4, 7a–e, 5, 8a–8e at 5.10 $^{-5}$ mol/L concentration, on SMPs previously contracted with CCh. The parameters (amplitude and frequency) after exposure to 1.10 $^{-7}$ mol/L CCh were taken as 100% and all subsequent responses were expressed as a percentage of this value. The grouping is by the type of substituent R_3: A (H); B (CH_3); C (C_6H_5); D($CH_2C_6H_5$); E(2-Cl-C_6H_4); F (CH(Cl)-C_6H_5).

Analysis of amplitude changes in spontaneous muscle contractions showed structure-dependent differences, well expressed when comparing the hybrid compounds according to the type of the second amide substituent **(R_3).** Some molecules demonstrated a weak effect, while others - significant inhibition. Hybrid molecules **4** and **5** showed over 50% reduction in amplitude, applied at a concentration of 5.10^{-5} mol/L. In this target group the most active amplitude relaxant is **3** with almost 90% inhibition. In contrast, compounds **7a** and **8a** (with **R_3 substituent** = **CH_3**) did not significantly affect the amplitude.

Favorites for medicinal candidates among newly synthesized diamide derivatives in the rest groups are respectively **8b (R_3 = C_6H_5); 6c** and **8c (R_3 = $CH_2C_6H_5$); 8d (R_3 = 2-Cl-C_6H_4); 8e (R_3 = CH(Cl)-C_6H_5),** which showed statistically significant inhibitory effect on the amplitude on abbreviation. According to the empirical discoveries, synthesized hybrid

molecules and their diamide derivatives manifest expressed biological activity, such as influence the processes on pharmacomechanical connection in typical for gastrointestinal tract (GIT) SM cells.

Grouping the compounds by the 2-phenylethylamine pharmacophore involved, the most potent effect we found at compounds **6b** and **6d** from first group (with 2-phenylethylamine), compound **4** from second group (with 3-chloro-2-phenylethylamine) and compounds **8d** and **8e** from third group (with 3,4-dimethoxyphenylethylamine), which can is explained by the presence on phenyl or benzyl anthranilic - related residue fragment. The rest compounds show relaxation influence on the spontaneous contractile activity with different mechanical strength on the observed reactions, but with an undeniable general antispasmodic character on the effect. Probably type on the substitutes and their position in the newly synthesized molecules are these specific structural elements that influence by different way affinity to the multitude receptors of SM and determine their efficiency as relaxants.

In this conntext, we can conclude that optimization of the chemical structure through suitable choice and placement of the substituents can lead to more powerful and selective medicinal candidates for therapy of complex diseases.

3.2. Experimental determination on anti-inflammatory activity

Inflammation represents pathological process resulting from from normal protected answer on organism at tissue damage caused from physical injuries, chemical substances or microbial agents. It is characteristic symptom at multitude chronic diseases and clinical is manifests through redness, swelling, warmth and pain. For suppression on inflammatory process often is use anti-inflammatory medicinal means from the groups on steroidal and non-steroidal anti-inflammatory (NSAIDs). The mechanism on action of NSAIDs are founded on inhibition the activity on the enzyme cyclooxygenase, key for synthesis on inflammatory mediators. Widely applied representatives as acetylsalicylic acid acid, ibuprofen and diclofenac are effective, but at long-term use can cause serious unwanted reactions, including gastrointestinal impairments, liver toxicity, ulcerations, hemorrhages, intestinal perforation and obstruction. These limitations emphasize the necessity from development on new compounds with better profile on safety and increased therapeutic efficiency.

In order to determine the anti-inflammatory potential of the compounds, we made assessment on their activity by two methods – *in vitro* and *ex vivo.*

3.2.1. *In vitro* determination inhibition denaturation on albumin

In vitro the analysis on anti-inflammatory activity was done through assessment on inhibition on thermally induced denaturation on albumin – a mechanism that is uses as model for research on anti-inflammatory action. Denaturation on proteins occurs as a result on changes in hydrophobic interactions, disulfide, electrostatic and hydrogen links. This one process leads to formation on autoantigens, which there is role in pathogenesis on different chronic and multifactorial inflammatory diseases, including IBS and IBD. In such cases inhibition on protein denaturation is considers for important indicator for potential anti-inflammatory activity.

The method used of inhibition The denaturation of human serum albumin upon heating is accessible and rapid. Through it are rated *in vitro* anti-inflammatory properties on hybrid

compounds grouped by the 2-phenylethylamine substituent as follows: I group (**3, 6a–6e)**, II group (**4, 7a–7e)** and III group (**5, 8a–8e)**. On Figure 9 results are presented as half from the maximum inhibitory concentration (IC $_{50}$). Like controls are used two NSAIDs – ASA and diclofenac. and one NSAIDs – prednisolone. For mebeverine was also used for comparison, which is uses as medication for relief on the symptoms of IBS, but our studies demonstrate and his *in vitro* and *ex vivo* potential anti-inflammatory properties that repeatedly exceed the used as standards anti-inflammatory medicinal. The drug Mebeverine has structural fragments, similar to the synthesized three groups of compounds, which streamlines the conclusions about the relationship between structure and biological properties.

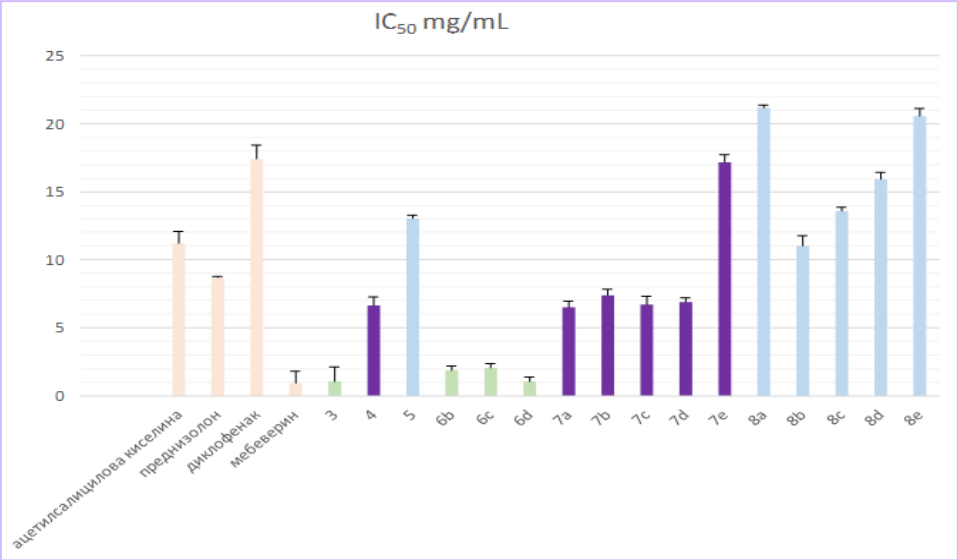


Figure 9. Inhibition of thermal denaturation of albumin by three groups* of compounds: *I – 3, 6b – 6c, II – 4, 7a – 7e and III – 5, 8a – 8e. Controls ASA, diclofenac, mebeverine and prednisolone were used for comparison. Results are expressed as IC$_{50}$.

In conclusion, we can summarize: The compounds from I group show highest anti-inflammatory activity comparable to that on the reference medicine Mebeverine. The introduction of chlorine and methoxyl substituents in the II and III group leads to downgrading of the activit, compared to I group and mebeverine, although in some casesit remains comparable or even higher than the effects of classic NSAIDs such as diclofenac. The compounds from the III group demonstrate effect similar to diclofenac, but weaker from ASA and prednisolone. Regardless from differences in activity, all they manifest promising ability for protection against thermal denaturation on albumin, which additionally confirms their therapeutic potential.

3.2.2. *Ex vivo* immunohistochemical assessment on anti-inflammatory activity.

Contractile activity and tone SM is modulate no only from calcium-dependent mechanisms, but also from pro-inflammatory mediators such as interleukin-1β (IL-1β) and the enzyme neuronal nitrogenous oxide synthase (nNOS). IL-1β, released from macrophages, stimulates the production on nitrogenous oxide (NO). This leads to increased NO concentration and decreased contractility of SM. It 's a process there is key meaning at inflammatory states where overproduction of NO causes hyporeflexia and functional violations on the bodies built from SM – gastrointestinal tract, circulatory system blood vessels and respiratory roads.

Immunohistochemical and enzyme-histochemical methods allow tracking on the expression of IL-1β, the activity on nNOS and the effects of NO, providing valuable information for pathogenesis on states as sepsis, inflammatory diseases on gut and autonomic dysfunctions.

To confirm received results from the previous ones research, we conducted additional immunohistochemical *ex vivo* tests for assessment the influence on hybrid molecules (**3–8e)** on the expression of IL-1β and nNOS in the SM P. These methods allow assessment on inflammatory and neuroregulatory mechanisms, key and in complex diseases as sepsis, IBD, IBS and autonomic dysfunctions. The done histological analysis after coloring for IL-1β and nNOS on paraffin cuts from stomach on rat shows clear distinguishable differences in dependence from the attached substance for incubation. The data from luminous microscopic measurements on intensity and density of IL-1β and nNOS the expression are summarized in Figure 10.

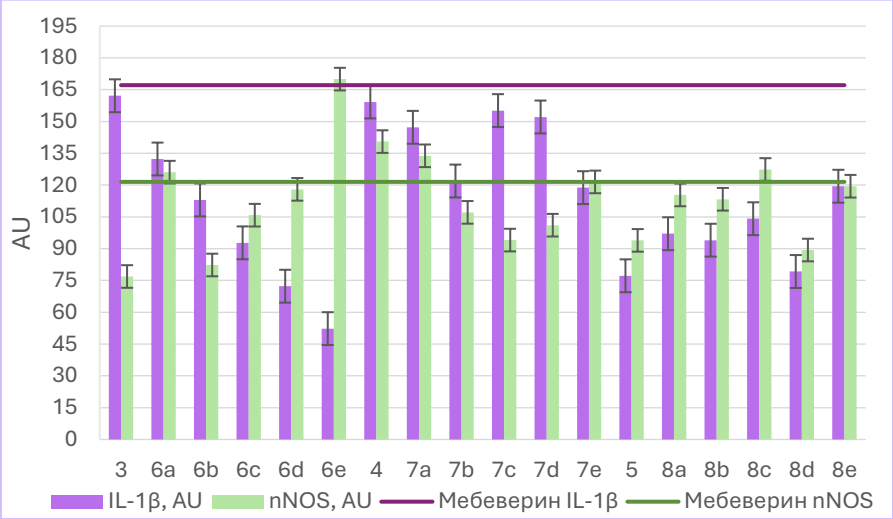


Figure 10. IL-1 β expression and nNOS in SM preparations incubated with hybrid compounds 3–8. The effects upon incubation with mebeverine are shown for comparison.

Regarding IL-1β expression in preparations incubated with each of the newly synthesized compounds, a decrease in expression was observed, comparable to or more significant than that induced by mebeverine. Compounds 5, 6d and 6e showed the most significant decrease in the intensity of the immunoreaction. This indicates that the synthesized hybrids suppress the expression of the potent pro-inflammatory cytokine IL-1β [358], affecting neuronal and SM cells.

In view of the obtained results of the immunohistochemical staining for the nNOS enzyme, the strongest modulatory effect on its expression was observed in the preparations incubated with the diamide derivative 6e, followed by those incubated with the hybrid 4 and its derivative 7a. This suggests a potential anti-inflammatory mechanism by stimulating neuronal-mediated release of NO. The experimental data highlight the potential of the hybrid compounds with the 3-chloro-2-phenylethyl substituent as candidates for the therapy of IBS, where restoration of normal NO signaling is of importance. The observed gradation in

activity among the three anthranilic hybrids also highlights the importance of the structure of the 2-phenylethylamine residue in modulating the biological effect.

The experimental results support the hypothesis that the hybrid compounds retain the anti-inflammatory activity characteristic of anthranilic acid, while revealing the modifying influence of additional pharmacophores, leading to potentiation or suppression of the effect.

3.3. Antimicrobial activity

Patients who suffer from IBS, often have bacterial overgrowth in the thin intestines. According to literature data, it is known that amides on anthranilic acid acid are very good antimicrobial agents. Regarding 2-phenylethylamines, moderate to severe toxicity has been reported. antimicrobial activity against some Gram-positive bacteria *(Bacillus subtilis, Mycobacterium smegmatis, Listeria monocytogenes* and *Staphylococcus aureus)* and moderate activity against yeast *Candida albicans.*

The synthesized three hybrids molecules and their fifteen diamide derivatives were applied in *in vitro* screening for detection their antimicrobial activity against human pathogenic bacterial and fungal strains. We used six Gram-positive bacteria, six Gram-negative bacteria, two yeast and five fungi. Based on the diameter of the zones on inhibition on bacterial and fungal growth caused from the new ones compounds, are conclusions made about the sensitivity or resistance of microorganisms presented in Table 3. The methanol used as solvent for the samples, no showed significant antimicrobial effect. At the same tested concentration as that of the new compounds, namely 1 mg/mL (0.1%), the antispasmodic Mebeverine does not show antimicrobial effect.

According to the experimental results obtained, one of the studied compounds (**4)** demonstrated promising antifungal activity against all tested mold strains – *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Rhizopus* sp. Moderate antimicrobial activity was also found against other microorganisms, including fungal strains such as *Mucor* sp., yeasts (*Candida albicans, Saccharomyces cerevisiae)*, as well as against Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Salmonella enteritidis, Salmonella typhimurium)* and Gram-positive bacteria (*Listeria monocytogenes, Enterococcus faecalis)*. Moderate sensitivity, characterized by inhibition zones with a diameter of 12–15 mm, was also reported for a number of derivatives of compound **4** – diamides **7a–e.**

The differences in antimicrobial activity against Gram-positive and Gram-negative bacteria are likely due to significant structural differences in their cell walls. Gram-negative bacteria possess an additional outer membrane rich in lipopolysaccharides, which significantly limits the penetration of hydrophobic and charged molecules. On the other hand, the cell wall of Gram- positive bacteria is thicker but devoid of an outer membrane, which usually facilitates the access of antimicrobial agents. The penetration of the compound can also be influenced by parameters such as lipophilicity, charge (including zeta potential) and molecular structure of the active substance. In this case, the presence of a 2-(3-chlorophenyl)ethylamine fragment and a chlorine atom in the structure of compound **4** and its derivatives **7a–e** probably contributes to their increased biological activity through improved penetration and interaction with microbial targets.

Table 3. Antimicrobial activity of the tested compounds.

Inhibited area,mm / Micro - organism	Tested compounds													Positive controls				
	3	**4**	**5**	**6b**	**6c**	**6d**	**7a**	**7b**	**7c**	**7d**	**7e**	**8b**	**8d**	**K**	**N**	**A**	**P**	**F**
***Bacillus subtilis* ATCC 6633**	R	**I**	–	–	R	–	R	R	R	R	R	–	R	**S**	NA	**I**	–	NA
***Listeria monocytogenes* NBIMC 8632**	–	**I**	–	–	–	–	R	**I**	–	R	R	–	–	**S**	NA	**S**	**I**	NA
***Enterococcus faecalis* ATCC 29212**	–	**I**	–	–	–	–	**I**	R	–	R	–	–	–	**S**	NA	**S**	–	NA
***Micrococcus luteus* 2YC-YT**	–	**S**	R	–	–	–	R	**I**	**I**	**I**	R	–	**I**	NA	NA	–	–	NA
***Salmonella enteritidis* ATCC 13076**	–	**I**	R	–	–	–	**I**	R	R	R	R	–	–	NA	NA	–	–	NA
***Salmonella typhimurium* NBIMCC 1672**	–	**I**	R	–	–	–	R	R	–	**I**	–	R	R	**S**	NA	**S**	–	NA
***Klebsiella pneumoniae* ATCC 13883**	–	R	R	–	–	–	**I**	–	–	R	**I**	**I**	–	**S**	NA	**S**	–	NA
***Escherichia coli* ATCC 25922**	R	**I**	R	–	R	–	R	R	R	**I**	R	R	R	**S**	NA	**I**	–	NA
***Pseudomonas aeruginosa* ATCC 9027**	R	**I**	R	–	R	–	R	R	R	R	R	R	R	NA	NA	**I**	–	NA
***Candida albicans* NBIMCC 74**	–	**I**	–	–	–	–	–	–	–	–	–	–	–	NA	**S**	NA	NA	–
***S. cerevisiae* ATCC 9763**	–	**I**	–	–	–	–	–	–	–	R	R	–	–	NA	**S**	NA	NA	–
***Aspergillus niger* ATCC 1015**	–	**S**	**I**	–	–	–	R	R	R	R	R	R	R	NA	**S**	NA	NA	**S**
Aspergillus flavus	–	**S**	–	–	–	–	R	**I**	R	R	R	–	–	NA	**S**	NA	NA	**S**
Penicillium chrysogenum	–	**S**	R	R	R	R	**I**	**I**	R	**I**	R	R	R	NA	NA	NA	NA	**I**
***Rhizopus* sp.**	–	**S**	R	–	–	–	R	R	R	R	R	–	R	NA	NA	NA	NA	–
***Mucor* sp.**	–	**I**	–	–	–	–	–	–	–	–	–	–	–	NA	NA	NA	NA	–

***Legend: S** – sensitive microorganisms, **I** – moderately sensitive, **R** – resistant, "**–**"– not registered effect; **NA** – not applicable; **K** – camistatin; **N** – nystatin; **A** – ampicillin; **P** – penicillin; **F** – fluconazole.*

The experimental results show, that compound 4 possesses most strongly expressed antimicrobial and antifungal properties among the tested hybrid molecules. These results determine it as a promising structure for future developments, including in the context on complex inflammatory diseases with microbial component.

3.4. *In vivo* study on memory functions at experimental animals

There are medicinal preparations that influence simultaneously contractions of SM and memory functions. This effect is explained by the fact that some neurotransmitters and receptors participate as in the regulation on muscle tone, as well as in cognitive processes. Mebeverine is a second-generation analogue of papaverine and belongs to to antispasmodics, but there is more specific mechanism on action, which it distinguishes from the classics phosphodiesterases inhibitors. Mebeverine blocked sodium channels in SM, which decreases the entry of Na^{+}, which prevents spasms. Indirectly inhibits calcium channels, decreases concentration on intracellular Ca^{2+}, and as a result relax intestinal muscles. Because of the action you mainly on the GIT, without affecting the normal one peristalsis, is suitable at treatment of IBS. At the same time time the medicine no penetrates easy through BBB and there is no direct effect on the CNS.

Guided from the principle for discovery on new drugs with better profile on safety, higher selectivity, more efficient action, reduction on the lateral effects, including cognitive violations that often accompany the use on some antispasmodics, we investigated the potential to affect cognitive function in experimental animals on three from substances (Figure 11) shown best activity at antispasmodic and anti-inflammatory activity.

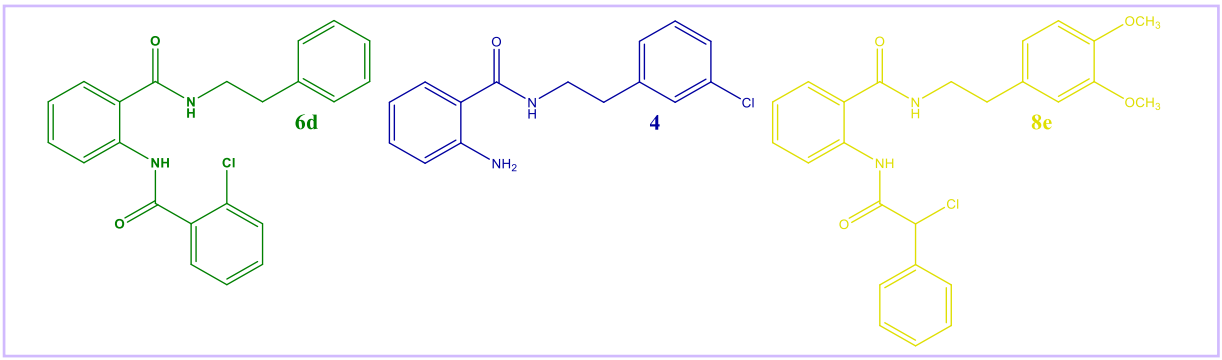


Figure 11. Structure on the selected compounds 6d, 4 and 8e for research the influence them over memory functions at experimental animals.

The experimental animals were divided into four groups, treated daily orally with a solution of the respective substance or physiological solution (Table 4).

Table 4. Experimental groups for research on motor activity and cognitive abilities on rats.

Group	Number animals	Treated with	Dose (per kg body weight) weight)
I	8	Physiological solution	1 mL/kg
II	8	6d	5 mg/kg
III	8	4	5 mg/kg
IV	8	8e	5 mg/kg

3.4.1. Activity cage test

At this test on first day the experimental group of rats treated with compound **4,** significant lower the horizontal motor activity compared to the control group from the same day (Figure 12). The others two groups of rats treated with substances **6 d** and **8 f** no changed essential number horizontal movements compared to the control group from 1st day.

At retest on the 11th day the control group significant lower number horizontal movements ($p < 0.05$) vs. first day on the same group. The rats treated with the three newly synthesized compounds they did number horizontal movements close to this one on the control group from the same day.

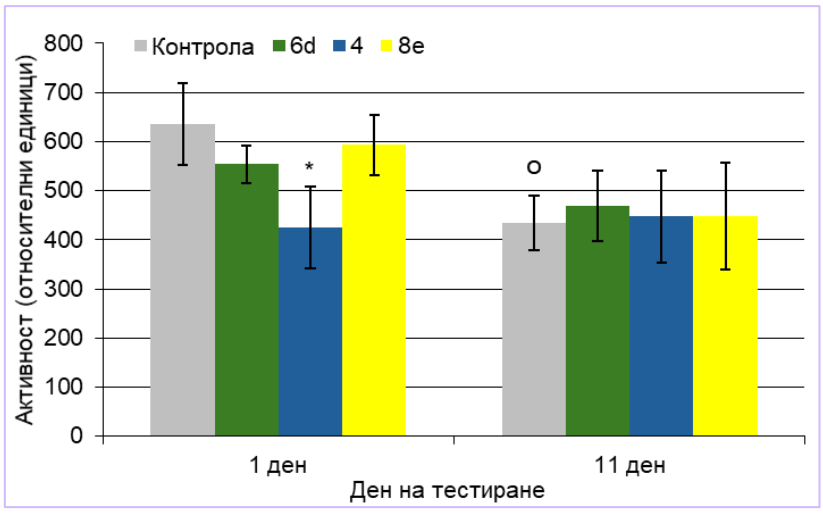


Figure 12. Horizontal motor activity – number of horizontal movements recorded in the Activity cage test.

Legend: *o = p<0.05 – comparison on the control group between the first and the mentioned day on testing; * = p<0.05 - comparison on the experimental control group group from the same day on testing.*

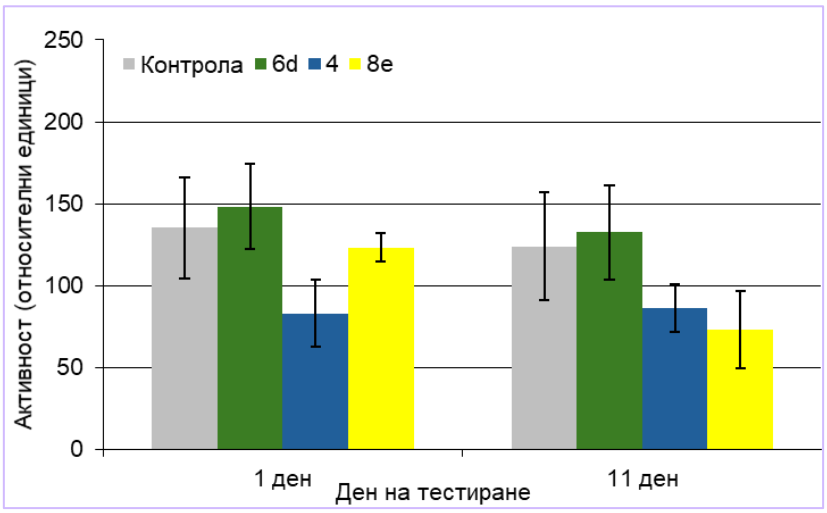


Figure 13. Vertical motor activity – number of vertical movements recorded in the Activity cage test.

The group treated with substance **6d,** do number vertical movements very close to this one on the control group. The rats treated with compounds **4** and **8e,** reduced number vertical movements as on the 1st day, and on the 11th day compared to the controls groups rats from the relevant days, but without statistical significance. By attitude on the vertical motor activity no is reports essential difference in number on the vertical movements at the control group as on the 1st and on the 11th day (Figure 1 3).

The received results at this one test allow the conclusion that newly synthesized substances no worsen the engine activity on the researched rats, they don't have muscle relaxant action on skeletal muscles, and also so no they show pronounced oppressive or exciting effect on the CNS.

3.4.2. Shuttle-box test

At *Shuttle box* the test for active training the control group rats significant raise number conditional responses (avoidances) to the 3rd ($p < 0.05$), 4th and 5th day training ($p < 0.01$), as well as at the test for memory ($p < 0.05$), compared to the 1st day (Figure 14). Rats treated with a substance **4,** reduced number avoidance on the 4th day training ($p < 0.05$) vs. the control group from the same day, but at the test for memory showed significant increase in their number ($p < 0.05$), compared to the control group from the 12th day. The experimental treated group with compound **8e** significantly reduce number conditional answers on the 3rd ($p < 0.05$), 4th and 5th day training ($p < 0.01$), as well as at the test for memory on the 12th day ($p < 0.05$), compared to the control group from the same days.

At the same test the control group increase number unconditional answers (escapes) to the 2nd, 3rd, 4th and 5th day training ($p < 0.05$), as well as at the test for long-lasting memory on the 12th day ($p < 0.05$), compared to the 1st day on the same group (Figure 15). The groups rats treated with the three newly synthesized compounds significant raised number escape room by time on the 5th days training ($p < 0.01$), as well as at the test for memory ($p < 0.01$), compared to the control group from the relevant days.

The three groups of rats treated with the newly synthesized compounds, significant raised number on between training sessions crossings in the 5 's days training ($p < 0.05$) and at the test for memory ($p < 0.05$), compared to the control group from the relevant days. The group that received substance **6d,** significant increase ($p < 0.01$) number between training sessions crossings on the 4th day training compared to the control from the same day. The rats treated with compound **4,** also significant increased number between training sessions crossings on the 5th day training ($p < 0.01$) vs. the control group on the same day (Figure 16).

At *Shuttle box* the test for active training with punishment reinforcement is found that the three compounds have different effects on cognitive functions learning and memory. Compound **8e** worsens as both training and long-term memory. The newly synthesized compound **4** no affects the training, but improves permanently. The substance **6d** does not affect the essential cognitive functions. All three investigated with connections raised the engine activity on animals, expressed in increments number between training sessions crossings as by time on the 5th days training, as well as the test for memory.

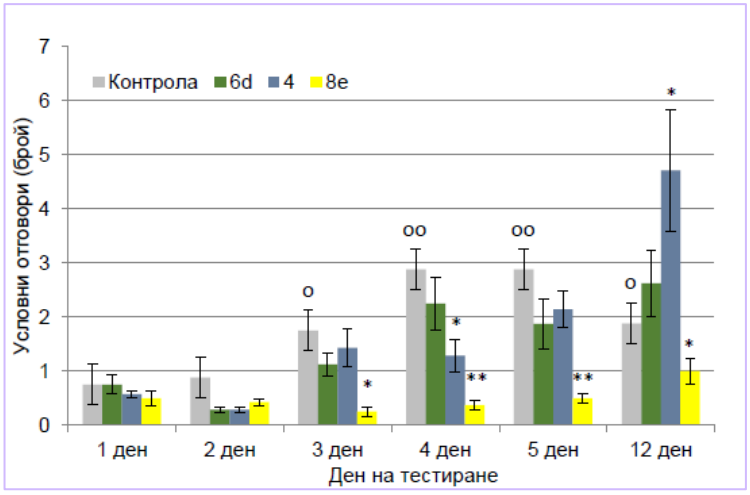


Figure 14. Shuttle-box test for active learning – number of conditional responses.

Legend: *o = $p < 0.05$ and oh = $p < 0.01$ – comparison on the control group between the first and the mentioned day on testing; * = $p < 0.05$ and ** = $p < 0.01$ – comparison on the experimental control group group from the same day on testing.*

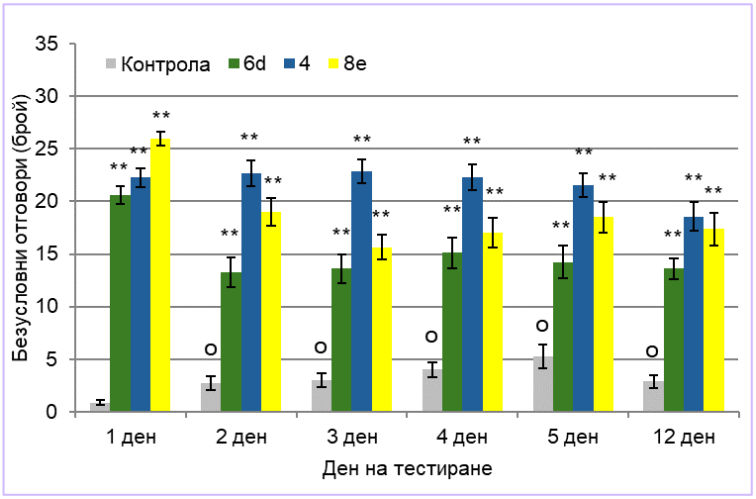


Figure 15. Shuttle-box test for active learning – number of unconditional responses.

Legend: *o = $p < 0.05$ – comparison on the control group between the first and the mentioned day on testing; ** = $p < 0.01$ – comparison on the experimental control group group from the same day on testing.*

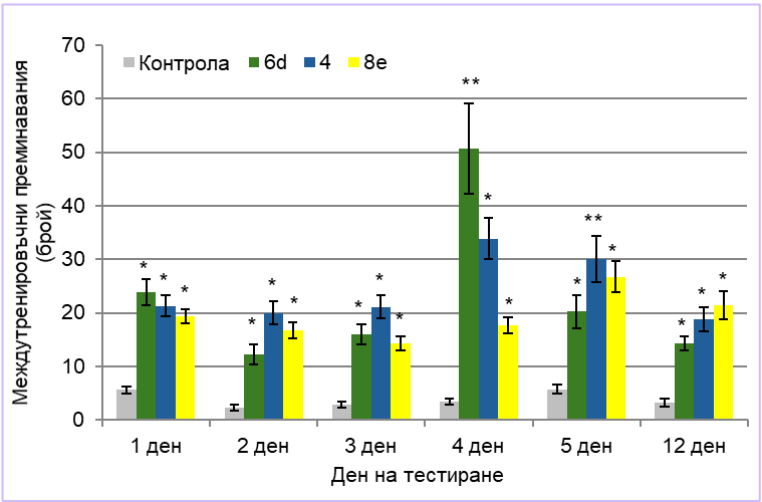


Figure 16. Shuttle-box test for active learning – number of inter-training passes.

Legend: ** = $p < 0.05$ and ** = $p < 0.01$ – comparison on the experimental control group group from the same day on testing.*

3.4.3. Step-through test

At *Step-through* the test for passive training the control group rats significant raised the latent time on stay in the light part on the apparatus on the 2nd day training, as well as in the tests for short-lived ($p < 0.05$) and long-lasting memory ($p < 0.05$), compared to the control group from 1st day (Figure 1 7). The groups animals treated with the three investigated compounds (**6d, 4, 8e)**, by time on the two-day educational session both the test for memory they did latency on the reactions very close to this one on the control group from the relevant days. Based basis on received results from *Step-through* the test for passive training with punishment reinforcement can be concluded that the three investigated compounds (**6d, 4, 8e)** no worsen the processes on learning and memory on rodents, showing results exceptionally close ones to these on the control group.

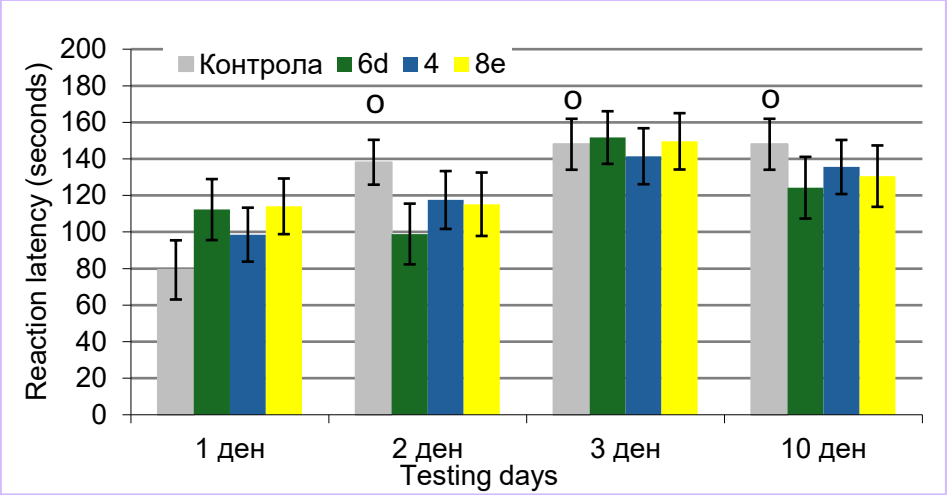


Figure 17. Step-through test for passive learning with penal reinforcement.

Legend: *o = p < 0.05 – comparison on the control group between the first and the specified testing day.*

3.4.4. Step-down test

At this one test for passive training the rats from the control group raised slightly, statistically insignificant, the latent time on stay on the vibrating platform on the apparatus by time on training, as well as in the two the test for memory, compared to the first day (Figure 1 8). Rats treated with a substance **4** significant increased the latent time on reaction on the 1st day training ($p < 0.05$), compared to the control from the same day. The group treated with compound **6d** increase significant latency on the reactions on the 2nd day training ($p < 0.05$) and at the two the test for memory, compared to controls rats from the same days. The rats that received compound **8e,** also showed increased latently time by time on training and tests for memory, but it was statistically insignificant.

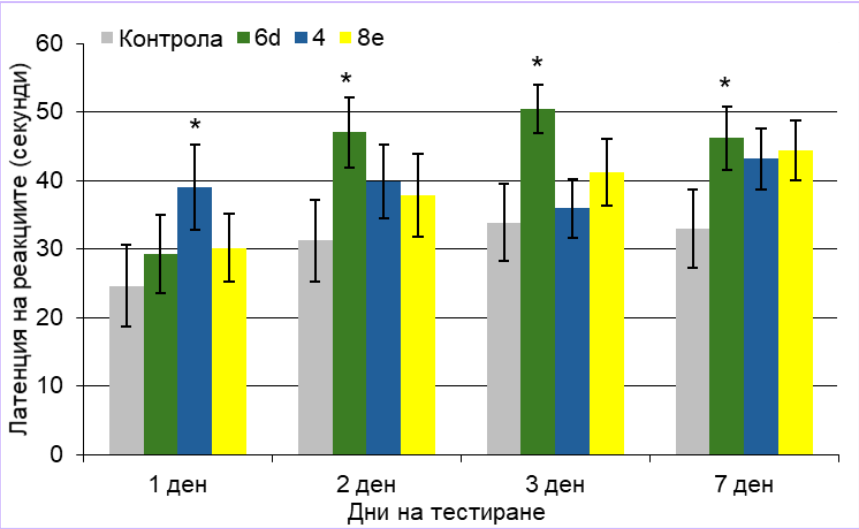


Figure 18. Step-down test for passive learning with penal reinforcement.

Legend: ** = $p < 0.01$ – comparison on the experimental group with the control group from the same day of testing.*

At *Step-down* the test for passive training with punishment reinforcement is found that substance **4** improves only the training, and the compound **6d** improves training, short-term and long-term memory. When this one test the compound **8e** no shows essential effect on cognitive functions.

In conclusion, the three investigated compounds no worsen cognitive functions and not suppress the locomotive activity on experimental animals. Compound 6d showed highest therapeutic potential, improving as training, both short-term and long- term memory. These results are best expressed in the Step-down test conducted last by order, which suggests accumulation on the compound and its effects after long-term application. This justifies the necessity from future research involving at least monthly in advance treatment with the same compounds, with the aim of comparison of the results obtained results with already available data.

CONCLUSIONS

1. For first road by the specified method with many good yields are received 18 hybrids on anthranilic acid, from which 16 new.

2. All synthesized compounds are purified, isolated and characterized by their temperatures on melting and spectral methods, in this number FT-IR, ^{1}H-NMR, ^{13}C-NMR, HRMS.

3. The antispasmodic effect was measured activity on all synthesized compounds, such as for five from them (**6b** and **6d** from first group, **4** from second group, **8d** and **8e** from third group) this activity is better compared to the known antispasmodic mebeverine.

4. Anti-inflammatory is evaluated activity on all synthesized compounds by two the method *in vitro* and *ex vivo.* All investigated compounds manifest promising protection against thermal denaturation on albumin, such as strongest activity demonstrate compounds **3** and **6d.** *Ex vivo* the research broadcast the hybrids **6e, 6d** and **5** as substances with essential inhibitory action on synthesis on pro-inflammatory cytokine IL-1β, and hybrids **6e, 4** and **7a** show highest activity compared to the expression on the enzyme nNOS.

5. Activity was investigated on three from compounds with many good antispasmodic activity at influence memory functions on experimental animals, such as one from them **(6d)** turned out significant effect in processes on memory and learning.

6. It has been established very good antimicrobial activity for compound **4** compared to pathogens mushrooms *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Rhizopus* sp. and moderate activity compared to yeast *Candida albicans* and *Saccharomyces cerevisiae.* The studied strains Gram-positive bacteria *Listeria monocytogenes, Enterococcus faecalis, Micrococcus luteus* and Gram-negative bacteria *Salmonella enteritidis, Salmonella typhimurium, Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* manifest moderate sensitivity by attitude on 3-Chlorophenylethylamine anthranilov hybrid **4** and diamide his derivatives **7a–f.**

7. Connection established structure – biological activity for each from the groups synthesized compounds.

CONTRIBUTIONS

SCIENTIFIC CONTRIBUTIONS

1. Design is done on small molecules as antispasmodics and are found synthetic approaches for receiving them.

2. It has been theoretically investigated the influence on antispasmodic activity from the introduction on different substitutes.

3. Found are clear dependencies between molecular structure and biological activity for synthesized compounds that can serve as basis for future rational design on biologically active compounds.

SCIENTIFIC AND APPLIED CONTRIBUTIONS

1. For first way, by means of suggestions synthetic method, are 18 compounds were obtained, from which 16 represent new structures, unpublished so far in the scientific literature.

2. For first time is measured antispasmodic activity on all 18 synthesized compounds and for five from them this one activity is better compared to the known antispasmodic mebeverine.

3. Degree proven on biological activity, characterizing one from the hybrids as substance with functional properties characteristics on antispasmodic with anti-inflammatory action, influencing memory functions at experimental animals.

PUBLICATION ACTIVITY

PUBLICATIONS IN REFEREED SCIENTIFIC JOURNALS

1. **Milusheva M,** Gledacheva V, Stefanova I, Feizi- Dehnayebi M, Mihaylova R, Nedialkov P, Cherneva E, Tumbarski Y, Tsoneva S, Todorova M, Nikolova S et al. *Synthesis, Molecular Docking, and Biological Evaluation of Novel Anthranilic Acid Hybrid and Its Diamides as Antispasmodics.* IJMS 2023, 24, 13855. **(Q1, IF 4.9)** https://doi.org/10.3390/ijms241813855

2. **Milusheva M,** Todorova M, Gledacheva V, Stefanova I, Feizi- Dehnayebi M, Pencheva M, Nedialkov P, Tumbarski Y, Yanakieva V, Tsoneva S, Nikolova S et al. *Novel Anthranilic Acid Hybrids—An Alternative Weapon against Inflammatory Diseases.* Pharmaceuticals 2023, 16, 1660. **(Q1, IF 4.3)** https://doi.org/10.3390/ph16121660

3. **Milusheva M,** Stoyanova M, Gledacheva, V Stefanova I, Todorova M, Pencheva M, Stojnova K, Tsoneva S, Nedialkov P, Nikolova S. *2-Amino-N-Phenethylbenzamides for Irritable Bowel Syndrome Treatment.* Molecules 2024, 29, 3375. **(Q1, IF 4.2)** https://doi.org/10.3390/molecules29143375

The presented scientific results have raised interest in the chemical research literature, objectively presented as **21** independent citations (until 2025) on the listed publications, discovered in *Scopus* and/or *Web of science* databases.

By data from *Scopus* the author's index on Hirsch (**h-index)** is **4.**

PARTICIPATION IN RESEARCH PROJECTS

1. The National program"Young scientists and postdoctoral students – 2"– module young scientists, selection 2022 from Pharmaceutical faculty, Medical University – Plovdiv, 2022–2023.

2. Project No. BG-175467353-2023-13-0075-C01 dated 15.12.2023 of topic"*Design, synthesis and biological activity on new hybrid molecules for treatment on dementia",* FNI Competition for financing on fundamental scientific research – 2023, with a supervisor Assoc. Prof. Dr. Stoyanka Atanasova, 2023–2026.

3. Project No. D23-HF-001 of topic"*Receive"on phytoalexins and their analogues with potential for application in plant breeding protection"*within on project DUEcoS at PU"Paisius"Hilendarski ", with a leader Assoc. Prof. Dr. Stella Statkova-Abeghe, 2023–2025.

4. Project No. KP-06-M 63/8 of 15.12.2022 on topic"*Ex vivo study"on new analogues on mebeverine",* FNI, Competition for financing on fundamental research on young scientists and postdoctoral fellows – 2022, thematic direction"Medical"sciences", with a supervisor Head Assistant Professor Dr. Mina Pencheva, 2022–2024.

5. Project No. MU-HF-017 The National program"Young scientists and postdoctoral students – 2"– module young scientists, selection 2024 from Chemical Faculty, Paisii University Hilendarski ", 2024–2025.

PRESENTATIONS AT SCIENTIFIC FORUMS

Participation in international conferences

1. **Miglena Milusheva,** Mina Todorova, Stoyanka Nikolova, *Novel anthranilic acid hybrids – an alternative weapon against inflammatory pathologies,* e-poster, 9th International Electronic Conference on Medicinal Chemistry (ECMC 2023), **MDPI online, 1–30.11.2023.**

2. **Miglena Milusheva,** Marinela Ivanova, Mina Todorova, Yulian Tumbarski, Stoyanka Nikolova, *Synthesis, In Silico, and In Vitro Evaluation of New Anthranilic Acid Hybrid with 2-(3-Chlorophenyl)ethylamine and Its Diamides,* oral presentation, IPSEC – 7 th International Conference on Medical and Health Sciences, **Ordu, Turkiye, 6–8.07.2023.**

3. **Miglena Milusheva,** Mina Todorova, Stoyanka Nikolova, *Computational methods for in silico prediction of chemical toxicity of 1,3-disubstituted 3,4-dihydroisoquinoline derivatives,* oral presentation, 4 th International Black Sea Modern Scientific Research Congress, **Rize, Turkiye, 6–7.06.2023.**

4. **Miglena Milusheva,** Snezhana Stoencheva, Mina Todorova, Mina Pencheva, Stoyanka Nikolova, *Design and synthesis of novel hybrid molecules as anticoagulants,* oral presentation, 3rd Eurasia International Scientific Research and Innovation Congress, **Ankara, Turkiye, 29–31.01.2024.**

5. **Miglena Milusheva,** Snezhana Stoencheva, Mina Todorova, Mina Pencheva, Stoyanka Nikolova Atanasova, *Design, Synthesis, and Biological Evaluation of New Hybrid Molecules as Anticoagulants,* poster, 4 th Molecules Medicinal Chemistry Symposium – Harnessing the Power of New Drug Modalities (MMCS 2024), **Barcelona, Spain, 24–26.04.2024.**

6. Kirila Stojnova, Mina Todorova, Vera Gledacheva, **Miglena Milusheva,** Iliyana Stefanova, Stoyanka Nikolova Atanasova, *Synthesis and Spasmolytic Activity of 2-Amino-N-(3- Chlorophenethyl) Benzamide and Its Metal Complexes,* poster, 4 th Molecules Medicinal Chemistry Symposium – Harnessing the Power of New Drug Modalities (MMCS 2024), **Barcelona, Spain, 24–26.04.2024.**

7. Mihaela Stoyanova, **Miglena Milusheva,** Mina Todorova, Stoyanka Nikolova, *Synthesis, in silico, and in vitro evaluation of novel mebeverine analogs,* oral presentation, Ahi Evran 4 th International Conference on Scientific Research, **Kirsehir, Turkiye, 28.04.2024.**

8. Vera Gledacheva, Mihaela Stoyanova, **Miglena Milusheva,** Iliyana Stefanova, Yana Pashkulova, Mina Todorova, Kirila Stojnova, Stoyanka Nikolova, *Spasmolytic activity of novel mebeverine derivatives,* poster, X th International Conference of Young Scientists, USB – Plovdiv, **Plovdiv, Bulgaria, 20–23.06.2024.**

9. Mihaela Stoyanova, **Miglena Milusheva,** Vera Gledacheva, Mina Todorova, Iliyana Stefanova, Stoyanka Nikolova, *Synthesis and spasmolytic activity of novel mebeverine derivatives,* oral presentation, ISARC 2. International Black Sea scientific research and innovation congress, **Trabzon, Turkey, 29–30.06.2024.**

10. **Miglena Milusheva,** *When 2 Becomes 1: Assembling Novel Drug Molecules for IBS Treatment,* oral presentation, The 9th International USERN Congress and Prize Awarding Festival. Medical University of Plovdiv, **Bulgaria 8–10.11.2024.**

Participation in national conferences

1. **Miglena Milusheva,** Vera Gledacheva, Spogami Khattak, Stoyanka Nikolova, *Application of computational methods for in silico prediction of chemical toxicity,* report, Conference Science and Youth 2023, MND "Asclepius", **Plovdiv, Bulgaria, 21–23.04.2023.**

2. **Miglena Milusheva,** Stoyanka Nikolova, *In silico methods for drug design and development of novel drug candidates,* report, 12th Chemistry Conference, **Plovdiv, Bulgaria, 13–14.10.2023.**

3. **Miglena Milusheva,** Vera Gledacheva, Iliana Stefanova, Maria Banyalieva (student) from FF, MU- Plovdiv), Stoyanka Nikolova, *Synthesis and antispasmodic activity on new hybrids on anthranilic acid acid and its diamides as antispasmodics,* report, Pharmacy science with a future, **Plovdiv, Bulgaria, 17–19.11.2023.**

4. Vera Gledacheva, **Miglena Milusheva,** Iliyana Stefanova, Mina Pencheva, Maria Banyalieva, Stoyanka Nikolova, *Newly synthesized hybrids on anthranilic acid with antispasmodic and anti-inflammatory activity,* poster, Pharmacy science with a future, **Plovdiv, Bulgaria, 17–19.11.2023.**

5. Mihaela Stoyanova, **Miglena Milusheva,** Vera Gledacheva, Iliyana Stefanova, Mina Todorova, Kirila Stojnova, Stoyanka Nikolova, *Novel hybrid molecules as powerful spasmolytics,* poster, Instrumental techniques and methods for chemical analysis – challenges and new solutions, **Plovdiv, Bulgaria, 05.06.2024.**

6. **Miglena Milusheva,** Mihaela Stoyanova, Vera Gledacheva, Iliyana Stefanova, Mina Todorova, Mina Pencheva, Kirila Stojnova, Slava Tsoneva, Stoyanka Nikolova, *Synthesis, spasmolityc activity and anti-inflammatory potential of some anthranilic acid hybrids,* report, Seventh Scientific Conference for Students, PhD Students and Young Scientists"Challenges in Chemistry", **Plovdiv, Bulgaria, 18–19.10.2024.**

7. Tedi Yordanova, **Miglena Milusheva,** Mihaela Stoyanova, Vera Gledacheva, Iliyana Stefanova, Mina Todorova, Mina Pencheva, Kirila Stojnova, Slava Tsoneva, Stoyanka Nikolova, *2-amino-N-phenethylbenzamides for irritable syndrome treatment,* poster, Seventh Scientific Conference for Students, PhD Students and Young Scientists"Challenges in Chemistry", **Plovdiv, Bulgaria, 18–19.10.2024.**

8. **Miglena Milusheva,** Mina Todorova, Kirila Stojnova, Vera Gledacheva, Mina Pencheva, Mihaela Stoyanova, Iliyana Stefanova, Stoyanka Nikolova, *Natural isoquinoline alkaloids affecting neurodegenerative diseases,* e- poster, National scientific conference on The Institute by neurobiology, Bulgarian Academy of Sciences, **Sofia, Bulgaria, 12–13.12.2024.**

ACKNOWLEDGEMENTS

I want to express my sincere gratitude and respect to my scientific supervisors for their professional dedication, mentorship, support and inspiring personal example.

I am grateful to the members of the Scientific Jury for their constructive critical comments and recommendations during the procedure.

I would like to thank to the professors at the Faculty of Chemistry at Paisii Hilendarski University of Plovdiv for opening the door to science to me, as well as to my science and mathematics teachers who ignited the spark of scientific curiosity in me.

I am grateful for the opportunity to begin my academic career and to carry out a significant part of the biological experiments at the Medical University of Plovdiv.

I thank my colleagues, who with their support and assistance contributed to my professional growth.

I would like to thank to my family from the bottom of my heart for their unconditional love, patience, and faith in me.

This work is a result not only from effort and knowledge, but also from patience, inspiration and humane support. From here onwards I will continue with the gained experience, as well as with the deep gratitude to the people with whom I had honor to share this road.