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NEW PROMISING AGENTS AGAINST COPD AND ASTHMA AMONG THE AMIDES OF 1-OXO-3-PHENYL-ISOCHROMAN-6-CARBOXYLIC ACID

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Background: Bronchodilators, which are compounds that can relax airway smooth muscle, are perhaps the most important component of combination therapy for chronic obstructive pulmonary disease, one of the most common non-communicable diseases in the world, which is the second most lethal disease after cardiovascular disease. Unfortunately, current clinical bronchodilators, whose activity is mediated by their interaction with muscarinic acetylcholine receptors, have side effects (up to myocardial infarction) due to their cross-affinity for different types of these receptors, including those prevalent in the heart muscle.
Objectives: The aim of this work is to search/develop compounds — effective bronchodilators capable of selectively inhibiting type 3 muscarinic acetylcholine receptors (M_3 receptors), predominantly present in smooth muscles and not characteristic of cardiomyocytes.
Materials and Methods: High-throughput virtual screening of a collection of 150,000 compounds was conducted on the spatial structure of the M_3 receptor, reconstructed in our previous studies. The effect of substances on contractile activity was investigated using tensometry in isometric mode on multicellular tracheal preparations. Antagonistic activity and type of inhibition were determined against the background of acetylcholine application (concentration range 10^{-10}–10^{-3} M). To establish the affinity value of the compound-antagonist, the Schild regression equation was used.
Results: Based on virtual screening data, a series of compounds — amides of 1-oxo-3-phenyl-iso-chroman-6-carboxylic acid — were selected for biological testing. For two of these compounds (Compounds 1 and 7), the ability to selectively inhibit M_3 receptors was demonstrated. Specifically, the affinity value pK_B for Compound 1 was 7.28 ± 0.70, with an IC_{50} of $5.25 \cdot 10^{-8}$ M. A critically important advantage of this compound is its ability, at equal concentrations, to more effectively inhibit signal transmission through M_3 receptors compared to ipratropium bromide — a clinical cholinergic receptor inhibitor.
Conclusions: The sufficient effectiveness of inhibition and significantly increased selectivity of the studied compounds specifically towards M_3 receptors provide strong grounds to consider these compounds as promising precursors of new generation cholinolytic drugs with targeted action on M_3-type cholinergic receptors.
KEY WORDS: chronic obstructive pulmonary disease (COPD); acetylcholine receptor; virtual screening; molecular docking; tensometry; selective M3 antagonists.

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In the human respiratory tract, M_2 and M_3 type cholinergic receptors play a direct role in regulating the lumen of the bronchial tree [1–4]. The physiological functions of M_2 cholinergic receptors, located in smooth muscle cells and postganglionic parasympathetic neurons, are to limit β_2-adrenoreceptor-mediated relaxation of smooth muscles by reducing intracellular cAMP concentration and to suppress the release of the neurotransmitter acetylcholine from nerve endings of postganglionic neurons [1, 5, 6]. Although M_2 type receptors significantly outnumber M_3 cholinergic receptors in smooth muscle cell membranes, the latter are functionally the main type of acetylcholine receptors that facilitate their contraction and narrowing of the respiratory tract lumen, as well as regulate the secretory activity of mucosal glands [1, 2, 7–9].

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are among the most common non-infectious diseases worldwide. According to data from the Global Asthma Network and the Global Initiative for Asthma, asthma affects 1 to 18% of the population in various countries, with a total global number of patients exceeding 300 million; a concerning global trend is the annual increase in patients diagnosed with asthma by 2.9% [9]. Asthma is characterized by transient airflow limitation in the airways, as well as airway hyperresponsiveness and sometimes remodeling of the airway wall tissues; these symptoms are also primary in COPD.

Pharmacological drugs used in the treatment of asthma and COPD are divided into several groups: long-acting β_2-adrenoceptor agonists (LABA), short-acting β_2-adrenoceptor agonists (SABA), long-acting muscarinic acetylcholine receptor antagonists (LAMA), short-acting muscarinic acetylcholine receptor antagonists (SAMA), as well as inhaled and oral corticosteroids.

Currently, the most commonly used drug in Ukraine for COPD and bronchial asthma with M-cholinolytic activity is ipratropium bromide [10, 11]. Like atropine, ipratropium is a competitive antagonist of muscarinic acetylcholine receptors. It has gained widespread use in the therapy of asthma and COPD, as unlike atropine, it does not cross the blood-brain barrier and is poorly absorbed through the gastrointestinal tract walls. Ipratropium is recommended by the Global Initiative for Chronic Obstructive Lung Disease 2009 (according to the Global strategy for diagnosis, management, and prevention of COPD) for four times daily inhalation use by COPD patients as a short-acting muscarinic antagonist (SAMA), with the maximum effect achieved within 60–90 minutes and the duration of therapeutic effect lasting 4–6 hours.

However, the optimal strategy for the therapy of obstructive phenomena in the airways with cholinolytic drugs (more precisely, drugs with properties of long-acting muscarinic antagonist — LAMA) involves the use of M_3-selective antagonists [1, 12]. Current COPD therapy protocols (USA, EU countries) and asthma include the use of such LAMA: tiotropium (recommended at a dose of 5 or 18 μg depending on the nebulizer functioning method, once daily) and aclidinium (recommended at a dose of 400 μg twice daily), glycopyrronium (recommended at a dose of 50 μg once daily), and umeclidinium (for COPD treatment and potential use in asthma treatment, recommended at a dose of 62.5 μg once daily) [2, 13]. According to a meta-analysis [14], for newer LAMA drugs (aclidinium, glycopyrronium, and glycopyrronium), no significant therapeutic advantages were found compared to the earlier tiotropium bromide (the only LAMA drug for COPD therapy until 2012).

Thus, currently, the active substance in drugs for the therapy of uncontrolled asthma is tiotropium bromide — a structural analog of ipratropium bromide, which is a functional antagonist of M_3 cholinergic receptors. This is because, although it binds to M_2 receptors, it dissociates from these significantly faster than from M_3 type receptors. Compared to ipratropium, tiotropium bromide has lower oral bioavailability and systemic side effects, and an extended half-life in patient's body [15–17]. However, the use of tiotropium bromide for the

therapy of stable asthma may be ineffective due to insufficient drug dosage, while increasing the dosage can lead to side effects [1, 18].

Therefore, the aim of our study was the directed search and biological testing of low-molecular-weight selective inhibitors of M_3 type muscarinic acetylcholine receptors.

MATERIALS AND METHODS

Virtual Screening

The predicted structure of the M_3-type muscarinic acetylcholine receptor [19] was used as a target for virtual screening. Two binding sites were identified. Binding site 1 (orthosteric) and binding site 2 (allosteric) utilizing the ICM Pocket Finder tool [20]. The first site is a combined pocket from the area located in a fold formed by helically twisted transmembrane domains, with which acetylcholine binds, as well as the area located higher, between the loops that bind the transmembrane domains. The second is on the reverse, intracellular part, formed by transmembrane domains. The orthosteric site has better characteristics than the allosteric one (larger volume of 1013.7 $Å^3$ compared to 461.8 $Å^3$ for the allosteric site, aromatic factor of 0.15 compared to 0, and drug-like index (DLID) [21] of 1.35 compared to 0.13). After detailed analysis and determination of the parameters of these sites, the orthosteric site was chosen for docking studies.

Virtual screening of the Department of Medicinal Chemistry Institute of Molecular Biology and Genetics NAS of Ukraine compounds collection (150,000 compounds) was conducted utilizing AutoDock 4.2 [22] molecular docking software with previously reported parameters and protocols [23]. 3,000 top-scored compounds (2% of all screened compounds) were selected according to AutoDock score for visual inspection and cherry-picking.

The BIOVIA Discovery Studio Visualizer [24] was utilized for visual inspection and ligand-target interaction analysis of top scored compounds. The parameters of the visualizer related to the construction of molecular bonds were used by default. The criteria for selecting compounds for pharmacological tests were as follows: 1. Compounds should bind to the acetylcholine-binding site; 2. Compounds should also bind to the subpocket above the acetylcholine-binding site for enhanced affinity and selectivity; 3. There must be at least one hydrogen bond.

Functional pharmacological studies of compound activity in vitro

Testing the effect of muscarinic acetylcholine receptor inhibitor substances was carried out on the contractile activity of the smooth muscles of the trachea using male Wistar rats (weighing within 230–250 g). Rats were euthanized under ether anesthesia, and all animal manipulations were conducted in accordance with the International Convention on the Work with Animals and the Law of Ukraine 'On the Protection of Animals from Cruel Treatment' (protocol of the meeting of the bioethics commission of the National Scientific Center 'Institute of Biology and Medicine' No. 3 dated May 2, 2019).

The study of contractile activity was conducted using tensometry in isometric mode on multicellular tracheal preparations, which were rings with an intact mucosal lining containing at least four cartilaginous rings. For the experiments, tracheal fragments from the beginning of the bifurcation to 1–1.5 cm above the bifurcation were used.

In the experiments, Krebs solution was used (mM): 120.4 NaCl; 5.9 KCl; 15.5 $NaHCO_3$; 1.2 NaH_2PO_4; 1.2 $MgCl_2$; 2.5 $CaCl_2$; 11.5 glucose; the pH of the solution was 7.4. Tracheal rings were placed in a working chamber (volume 2 ml) bubbled with an oxygenated gas mixture of Krebs solution (95% O_2/5% CO_2, flow rate — 5 ml/min), and thermostated at 37°C. The tracheal preparations were given a passive tension of 10 mN and left for 1 hour; the study began

after recording several reproducible contractile reactions to the application of hyperkalemic solution and acetylcholine (10^{-6} M). The contractile activity was investigated using a capacitive force sensor; the amplified signal was recorded using an ADC.

Preliminary screening of compounds for their ability to inhibit muscarinic cholinergic receptors was conducted by recording contractile responses of tracheal preparations to the application of acetylcholine (10^{-5} M) under the condition of prior action of the tested compounds (at a concentration of 10^{-4} M) for 10 minutes.

To study the antagonistic activity of muscarinic cholinergic receptor antagonists (ipratropium bromide and tested substances), contractile responses of tracheal preparations to the application of acetylcholine at a concentration of 10^{-4} M were recorded under cumulative increases in antagonist concentrations (10^{-10}–10^{-5} M, with the antagonist's prior action time of 10 minutes) and concentration-effect curves were constructed, from which the IC_{50} value was determined; the maximum contractile response (100%) was considered as the contraction to the application of acetylcholine without an antagonist.

To confirm the competitive type of inhibition by ipratropium bromide and tested substances, as well as to study their functional antagonism, concentration-effect curves of acetylcholine were recorded (acetylcholine concentration range 10^{-10}–10^{-3} M). Subsequently, the Schild regression equation was used to determine the affinity of the compound-antagonist. According to the Schild method, the antagonist-induced parallel shift of the concentration-effect curves was determined as the ratio of equieffective concentrations (CR) of the agonist in control and in the presence of an antagonist. From the Schild plots, the functional antagonism index (affinity index of the competitive antagonist) pK_B and the tangent of the slope angle were determined [25–27].

To study the cellular mechanisms of action of the compounds, contractile responses of the trachea to the application of the selective $mAChR_3$ agonist cevimeline (10^{-4} M), effectors of adrenoreceptors (adrenaline, 10^{-5} M; propranolol, 10^{-5} M; isoproterenol, 10^{-5} M), and nicotinic cholinergic receptors (nicotine, 10^{-4} M), blocker of inositol 1,4,5-trisphosphate-sensitive (IP_3) Ca^{2+} channels of the sarcoplasmic reticulum (2-APB, 10^{-4} M), and phospholipase C inhibitor (U-73122, 10^{-6} M) were recorded.

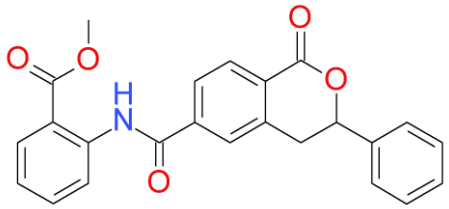
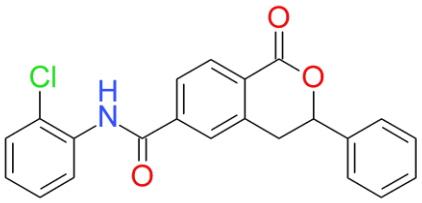
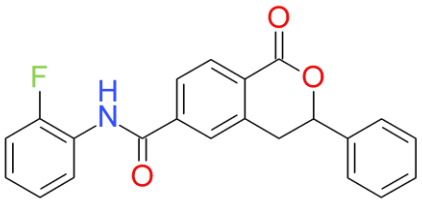
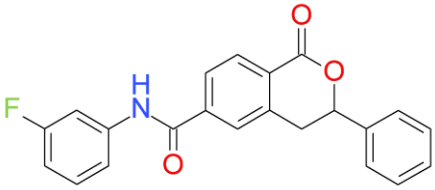
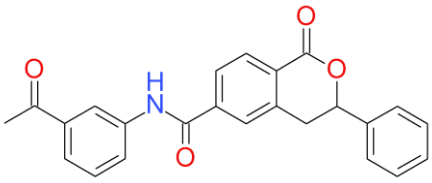
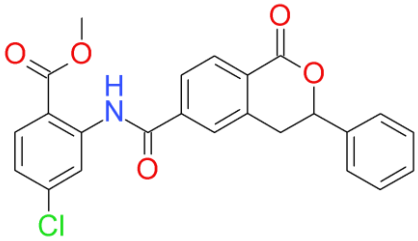
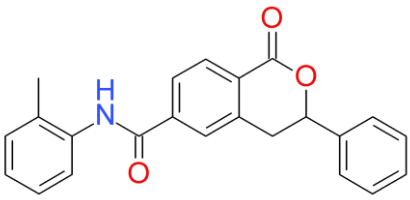
All compounds under study were initially dissolved in DMSO and introduced into the smooth muscle preparations in such a way that the final concentration of DMSO was 0.1%. Additionally, all experiments were conducted in the presence of DMSO in the bathing solution at a concentration of 0.1%.

Experimental data were analyzed using Origin Pro 2018 software. The samples were tested for their adherence to normally distributed populations using the Shapiro-Wilk criterion. To determine significant differences between the mean values of the samples, a paired t-test was used; multiple comparisons were conducted using one-way analysis of variance (ANOVA). In all cases, results were considered significant if $p < 0.05$. The significance of data approximation by a linear function was analyzed using Fisher's F-criterion; the coefficients of determination (R^2) were not less than 0.9. Results are presented as mean ± standard error of mean (SEM), n – number of experiments.

RESULTS AND DISCUSSION

For pharmacological testing *in vitro* on multicellular smooth muscle preparations of rat tracheal rings, seven most promising compounds — amides of 1-oxo-3-phenyl-isochroman-6-carboxylic acid (**1**–**7**, Table 1) with predicted cholinolytic activity were selected through preliminary *in silico* screening. As a target for screening, the spatial structure of the M_3 type cholinergic receptor, reconstructed in our previous studies [19], were used. Structure of these compounds are presented in Table 1.

Table 1. Structures and physico-chemical properties of the studied amides of 1-oxo-3-phenyl-isochroman-6-carboxylic acid

Compound	Structure
1	
2	
3	
4	
5	
6	
7	

In the first series of experiments, the reaction of smooth muscle preparations (SMP) of the trachea to the application of acetylcholine (10^{-5} M) was tested under the condition of prior

action of the tested compounds (all at a concentration of 10^{-4} M) for 10 minutes. Thus, two compounds were identified with the ability to suppress acetylcholine-induced contractions, identified as **1** and **7** (Fig. 1).

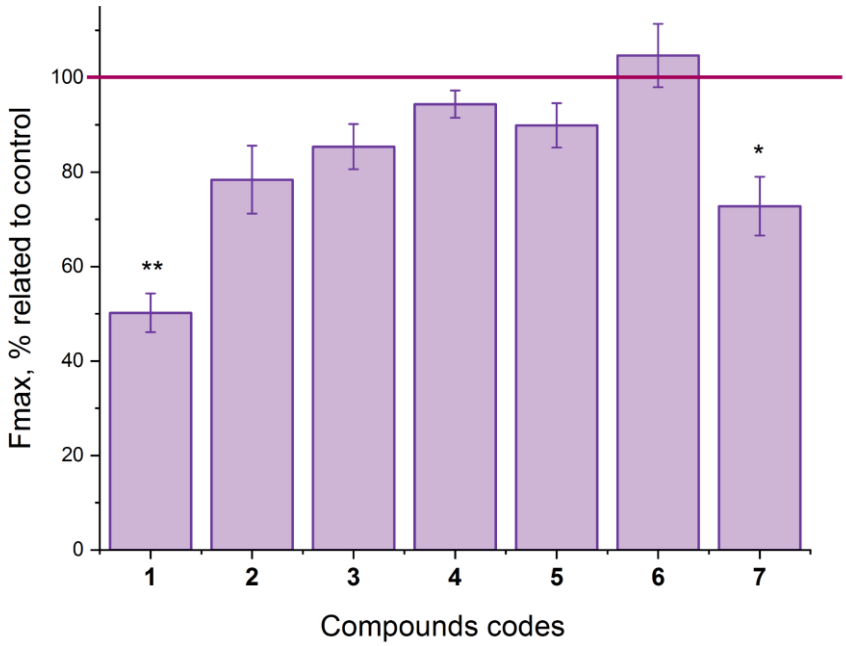
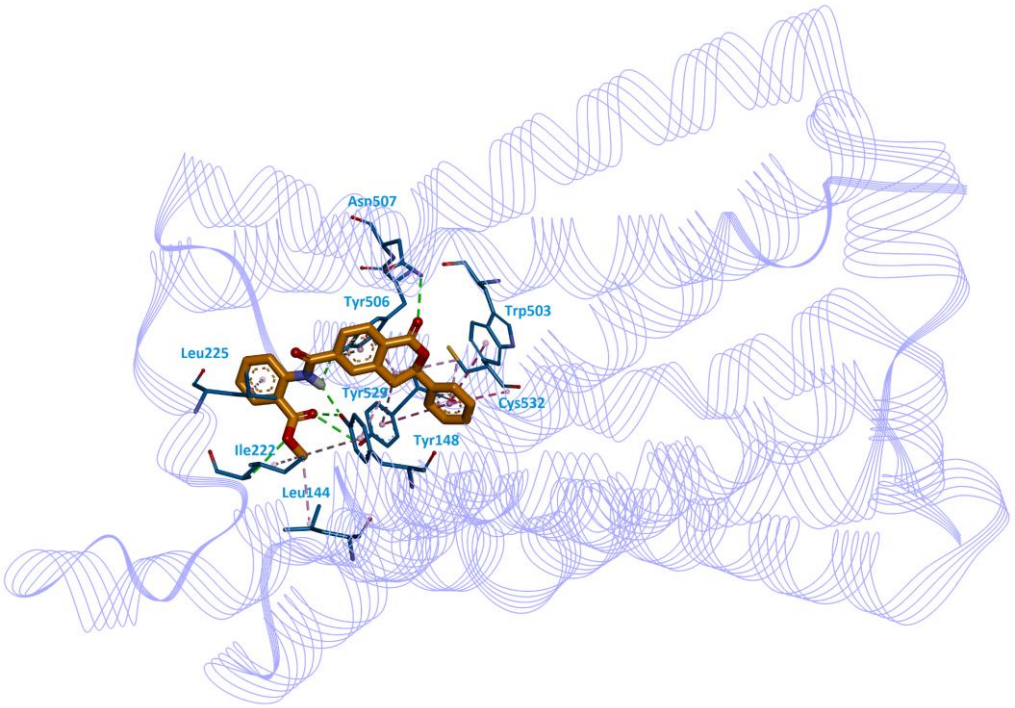


Fig. 1. Changes in the amplitude (F_{max}) of acetylcholine-induced (10^{-5} M) contractions of rat tracheal preparations by compounds (used at a concentration of 10^{-4} M, pre-incubation time 10 min).
Control values were taken as 100% (n = 7).
** — $p < 0.01$ and * — $p < 0.05$ — significant difference compared to control.

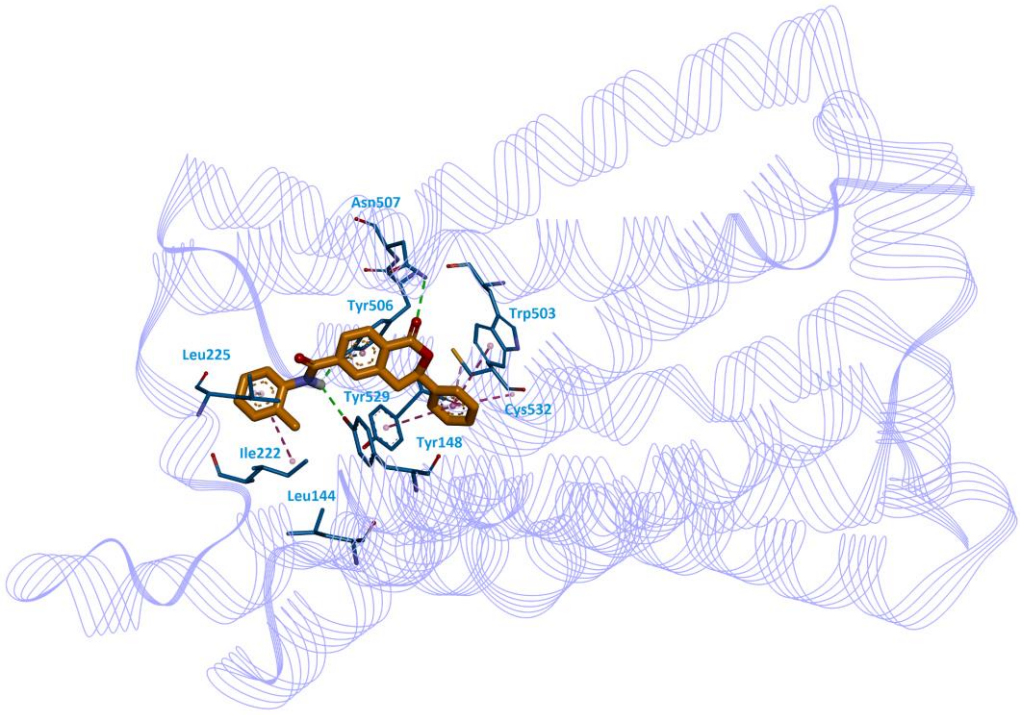
Spatial structures of the corresponding complexes of active compounds are depicted in Fig. 2. Both compounds interact with the M_3 cholinergic receptor in the same manner. The 1-oxo-3-phenyl-isochroman moiety interacts within the acetylcholine binding site, specifically forming a series of hydrophobic interactions with Tyr148, Trp503, Tyr506, Tyr529, Cys532, and a hydrogen bond with Asn507. The NH of amide group forms two hydrogen bonds with Tyr148 and Tyr506. The o-tolyl group of compound **7** engages in hydrophobic interactions with Ile222 and Leu225. Methyl o-benzoate group of compounds **1** has additional hydrophobic interactions with Leu144 and Tyr148, as well as hydrogen bonds with Tyr148, Ile222, and Tyr529.

In the subsequent series of experiments, acetylcholine-induced contractions of rat tracheal preparations were recorded under the action of selected compounds **1** and **7** (all used at a concentration of 10^{-4} M) against the background of prior incubation of SMP with the known cholinergic receptor antagonist ipratropium (used at a concentration of 10^{-6} M). Since the tested compounds did not affect the baseline tension and contractile response of SMP in the presence of ipratropium, it can be asserted that these substances are tropic to muscarinic cholinergic receptors.

The mentioned compounds did not affect the SMP responses to the application of the nicotinic cholinergic receptor agonist nicotine (used concentration 10^{-6} M), supporting the hypothesis of the selective action of the tested substances specifically on muscarinic acetylcholine receptors [21].



A



B

Fig. 2. Molecular complexes of M_3 cholinergic receptor with compound **1 (A)** and compound **7 (B)** obtained by molecular docking. Hydrogen bonds are shown by the green dotted lines, hydrophobic interactions are indicated by magenta dotted lines.

It was also established that both compounds and the known non-selective inhibitor of muscarinic cholinergic receptors, ipratropium bromide (in all cases used at a concentration of 10^{-6} M, with a pre-incubation duration of 10 minutes in the presence of the tested substance), could inhibit the contractions of SMP of the trachea activated by the selective M_3 cholinergic

receptor agonist cevimeline (fixed concentration of 10^{-4} M). The greatest inhibitory properties against M_3 cholinergic receptors were observed for compound **7** — under these conditions, cevimeline-induced contraction was 24.5 ± 3.6% relative to the control, taken as 100% (n = 7, p < 0.001). For comparison, a similar effect on cevimeline-induced SMP tracheal contractions for ipratropium bromide was registered, a compound that is the active ingredient in medicinal products for the therapy of bronchial asthma and COPD. It was found that ipratropium bromide at a concentration of 10^{-6} M causes an average inhibition of cevimeline-induced contraction to 63.2 ± 5.1% relative to the control, taken as 100% (n = 7, p < 0.01).

Subsequently, for the compound **7**, 'acetylcholine concentration-effect' curves were registered and analyzed, the type of inhibition, affinity indicators, and IC_{50} were determined. It was found that the slope of the Schild regression line (0.88 ± 0.12, coefficient of determination $R^2 = 0.98$) (Fig. 3, 4) indicates a competitive mechanism of action of this substance. The affinity value of this compound pK_B was 7.28 ± 0.70 and $IC_{50} = 5.25 \cdot 10^{-8}$ M.

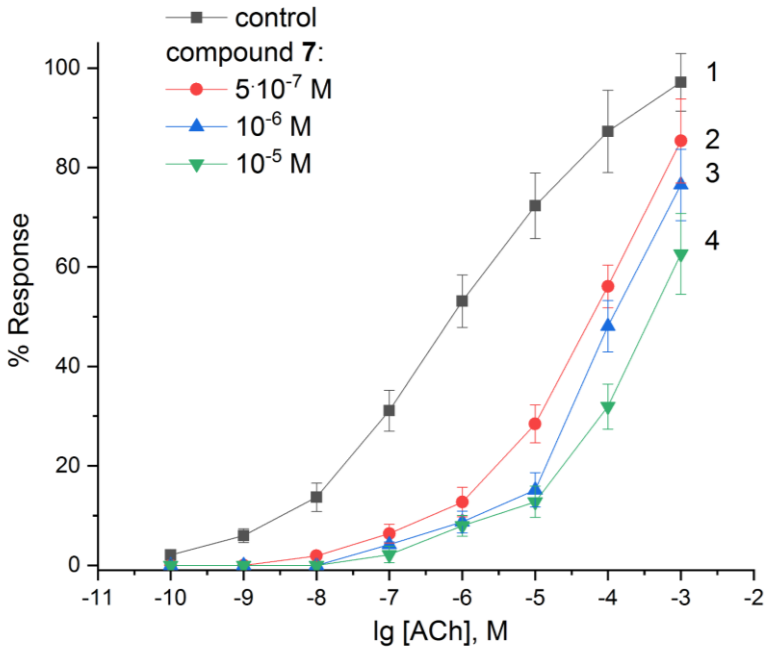


Fig. 3. 'Acetylcholine concentration-effect' curves in the presence of compound **7** for the activation of smooth muscle contractions of rat trachea: curve 1 — control (concentration range 10^{-10}–10^{-3} M), curves 2–4 — against the background of **7** at concentrations of $5 \cdot 10^{-7}$ M, 10^{-6} M, and 10^{-5} M, respectively. The amplitude of contractions under the action of antagonists is recalculated in % compared to acetylcholine-induced contraction in control (10^{-4} M), taken as 100%. Data are presented as M ± SEM, n = 7.

It has also been established that compound **7** does not significantly affect the nicotinic cholinergic receptors and adrenergic receptors of the respiratory tract in rats. Using a phospholipase C inhibitor (U-73122) and blockers of inositol 1,4,5-trisphosphate-sensitive (2-APB) and ryanodine-sensitive (caffeine) Ca^{2+} channels of the sarcoplasmic reticulum, it was demonstrated that the aforementioned compounds act on the intracellular signaling cascade through M_3 type muscarinic cholinergic receptors [28–31].

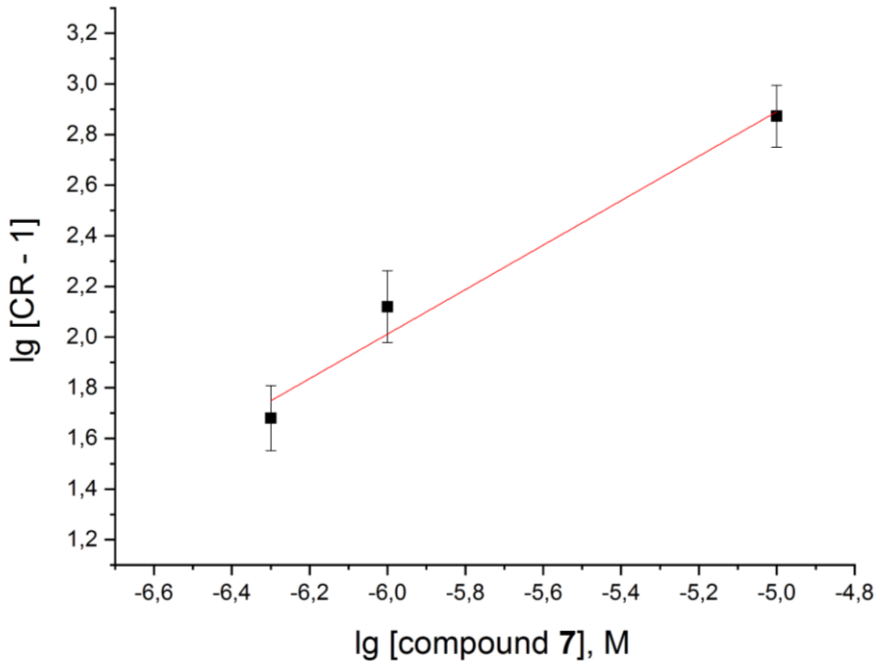


Fig. 4. Schild plot for the action of compound **7**. Data are presented as M ± SEM, n = 7.

Previously, we established [19] that for the known non-selective competitive antagonist of muscarinic cholinergic receptors, ipratropium bromide, the slope of the Schild regression line was 0.79 ± 0.07 (coefficient of determination R^2 = 0.99), and the affinity value pK_B was 9.14 ± 0.62 with an IC_{50} of $7.24 \cdot 10^{-10}$ M. Therefore, compound **7** has a lower affinity and is characterized by higher EC_{50} values compared to ipratropium bromide. However, a critically important advantage of compound **7** is its ability, at equal concentrations, to more effectively inhibit signal transmission through M_3 cholinergic receptors compared to ipratropium bromide.

CONCLUSIONS

Biological testing was conducted on smooth muscle preparations (SMP) of the trachea for compounds in the group of amides of 1-oxo-3-phenyl-isochroman-6-carboxylic acid with predicted inhibitory activity towards mAChRs: their pharmacological effects and parameters were studied. It was found that the compound **7** effectively inhibits (with an average IC_{50} value of $5.25 \cdot 10^{-8}$ M) and at equal concentrations significantly more inhibits signal transduction specifically through M_3 cholinergic receptors compared to ipratropium bromide, without having a significant effect on M_2 cholinergic receptors. Therefore, there are all reasons to consider these compounds as promising precursors of new generation cholinolytic drugs with targeted action on M_3 cholinergic receptors.

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CONFLICT OF INTEREST

The authors report that there is no conflict of interest.

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НОВІ ПЕРСПЕКТИВНІ ЗАСОБИ ПРОТИ ХОЗЛ ТА АСТМИ СЕРЕД АМІДІВ 1-ОКСО-3-ФЕНІЛ-ІЗОХРОМАН-6-КАРБОНОВОЇ КИСЛОТИ

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Актуальність. Бронходилататори — сполуки, здатні розслабляти гладеньку мускулатуру повітроносних шляхів, є чи найважливішим компонентом комбінованої терапії хронічного обструктивного захворювання легень — одного з найбільш поширених у світі неінфекційних захворювань, що займає друге місце за летальністю після серцево-судинних захворювань. На жаль, сучасні клінічні бронходилататори, чия активність опосередкована їх взаємодією з мускариновими рецепторами ацетилхоліну, мають побічні ефекти (до інфаркту міокарда) внаслідок їх перехресної спорідненості до різних типів цих рецепторів, зокрема, і до тих, що розповсюджені в серцевому м'язі.

Мета роботи: пошук/розробка сполук — ефективних бронходилататорів, здатних селективно інгібувати мускаринові рецептори ацетилхоліну типу 3 (M_3-рецептори), які представлені переважно в гладеньких м'язах і не характерні для кардіоміоцитів.

Матеріали і методи. Високопродуктивний віртуальний скринінг колекції 150000 сполук було здійснено щодо просторової структури М3-рецептора, реконструйованого в наших попередніх дослідженнях. Вплив речовин на скорочувальну активність досліджували методом тензометрії у ізометричному режимі на мультиклітинних препаратах трахеї. Антагоністичну активність і тип інгібування визначали на фоні аплікування ацетилхоліну (діапазон концентрацій 10^{-10}–10^{-3} М). Для встановлення величини афінності сполуки-антагоніста використовували рівняння регресії Шілда.

Результати. За даними віртуального скринінгу було обрано для біологічного тестування ряд сполук — амідів 1-оксо-3-феніл-ізохроман-6-карбонової кислоти. Для двох із них (сполуки 1 і 7) було продемонстровано здатність селективно інгібувати M_3-рецептори. Зокрема, велична афінності pK_B для сполуки 1 становила 7,28±0,70, а $IC_{50}=5{,}25\cdot10^{-8}$ М. Надзвичайно важливою перевагою цієї сполуки є її здатність за однакових концентрацій достовірно ефективніше пригнічувати проведення сигналу через M_3-рецептори порівняно з іпратропієм бромідом — клінічним інгібітором холінорецепторів.

Висновки. Достатня ефективність інгібування і значно підвищена селективність досліджених сполук саме стосовно M_3-рецепторів дають всі підстави вважати зазначені сполуки перспективними попередниками холінолітичних препаратів нового покоління зі спрямованою дією на холінорецептори M_3-типу.

КЛЮЧОВІ СЛОВА: хронічне обструктивне захворювання легень (ХОЗЛ); мускариновий ацетилхоліновий рецептор; віртуальний скринінг; молекулярний докінг; тензометрія; селективні М3-антагоністи.