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Reviewer's Report on the Ph.D. Dissertation Thesis of Agnieszka-Olejarz-Maciej entitled „ Multidirectional activity of new histamine and adenosine receptor ligands" performed under the direction of Prof. Katarzyna Kieć-Kononowicz as supervisor and Dr. Tadeusz Karcz as assistant supervisor

For many years, the Department of Technology and Biotechnology of Drugs of the Faculty of Pharmacy of the Jagiellonian University has been conducting research into the design and synthesis of potential ligands capable of simultaneously modifying the activity of several pharmacological targets in the CNS.

The main goal of the doctoral dissertation submitted for review was to design and perform tests of selected groups of histamine H_3 and H_4 receptor ligands in terms of their impact on several possible secondary messengers (cAMP, Ca^{2+}, β-arrestin) and to perform a the structure-activity relationship, based on which it was possible to indicate the structural fragments responsible for their functional selectivity. In addition, the Ph.D. student carried out tests on two groups of compounds - histamine H_3 receptor ligands and adenosine A_1 and A_{2A} receptor ligands - for possible MAO-B inhibition.

Histamine plays an important role in a variety of pathophysiological conditions. In peripheral tissues, histamine is mainly stored in mast cells and basophils. In allergic conditions, histamine is released from these cells and is responsible for several of the well-known symptoms of allergic conditions of the skin and airways. In the gastric mucosa, gastrin-induced histamine release fulfills an important physiological role by stimulating parietal cells to secrete gastric acid. In the CNS, histamine is synthesized in specific neurons that are localized in the tubero-mammillary nucleus of the posterior hypothalamus. These neurons project to all major brain areas and are involved in a variety of important physiological functions, including the regulation of the sleep-wake cycle, food intake, cardiovascular control, regulation of learning, and memory.

Histamine exerts its action via at least four distinct receptor subtypes (H_1, H_2, H_3, and H_4). All histamine receptors belong to the large family of G protein-coupled receptors. The H_3 receptor was originally described as an autoreceptor, that inhibits the release of histamine from histaminergic neurons in the brain. The inhibitory effect is due to the constitutive activity of the H_3 receptor. Furthermore, it has been shown that the H_3 receptor regulates the release of several important neurotransmitters (e.g. acetylcholine, dopamine, GABA, norepinephrine, serotonin), in both the peripheral and central nervous systems. The ligands of this receptor are currently being assessed for their clinical potential in allergy, inflammatory disorders, attention deficit hyperactivity disorder, Alzheimer's disease, Parkinson's disease, and obesity. Recently, several compounds have been described showing promising dual-directional activity not only against histaminergic H_3/H_1, H_3/H_2, or H_3/H_4 receptors but also H_3 with non-histaminergic G protein-coupled receptors and H_3 with serotonin reuptake inhibitors and H_3 with enzyme inhibitors (HMT, ACHE, BuCHE, etc.), which may prove to be useful therapeutics in the treatment of various CNS disorders after undergoing clinical trials.

The presence of H_4 receptors was demonstrated on mast cells and basophils suggesting their involvement in the development of inflammation. They have also been discovered in the mechanisms of allergic reactions. It has also been shown that these receptors are involved in

the phenomenon of mast cell chemotaxis and changes in the shape of eosinophils, which, together with mast cells and basophils, control the development of allergy and asthma. Many H_3 receptor antagonists and agonists also bind to the H_4 receptor, because of the high homology to the H_3 receptor, which is 58% for the transmembrane regions, and the 35% similarity. Like the H_3 receptor, histamine H4 receptors are protein-coupled Gi/o. Receptor stimulation causes a decrease in the concentration of cyclic adenosine-3′,5′-monophosphate (cAMP), leading to activation of mitogen-activated protein kinase (MAPK) and increased Ca^{2+} release.

Adenosine acts on specific receptors located on the surface of the neuronal membrane cell. To date, four types of adenosine receptors have been described (A_1, A_{2A}, A_{2B}, and A_3) and their main function is to control the excitation of nerve cells and regulate the secretion of other neurotransmitters. They are all coupled to a G-protein, through which they affect adenylyl cyclase, stimulating or inhibiting its activity. Adenosine is also important as a neurotransmitter, regulating the activity of neurons, the release of other neurotransmitters into synapses, and influencing their interactions with specific receptors. Adenosine receptors have traditionally been classified according to their differential coupling to adenylyl cyclase to regulate cyclic AMP levels. A_1 and A_3 receptors are coupled to $G_{i/o}$ proteins, whereas A_{2A} and A_{2B} receptors are coupled to $G_{s/olf}$ proteins. Therefore, activation of A_{2A} and A_{2B} receptors increases cyclic AMP production, leading to activation of protein kinase A (PKA) and phosphorylation of cyclic AMP binding protein (CREB). However, activation of A_1 and A_3 receptors inhibits cyclic AMP production and reduces PKA activity and CREB phosphorylation. In the CNS, A_1 receptors are widely distributed in neurons in the cerebral cortex, hippocampus, and cerebellum. In neurons, A_1 receptors are located in synaptic regions where they modulate the release of neurotransmitters such as glutamate, acetylcholine, serotonin, and GABA. A_{2A} receptors have a more restricted localization in the striatum and olfactory bulb. These receptors are present in neurons, microglia, and oligodendrocytes. The presence of A_{2A} receptors has been described in dendritic spines and postsynaptic regions of the basal ganglia. These receptors are located in presynaptic regions (in the hippocampus), where they modulate the release of neurotransmitters such as glutamate, acetylcholine, GABA, and norepinephrine. A_{2B} receptors are expressed at low levels on neuronal and glial cells such as microglia and astrocytes. Low levels of A_3 receptors are detectable in the hippocampus, cerebral cortex, cerebellum, and striatum, with cellular localization in neurons, astrocytes, and microglia.

Monoamine oxidases (MAO) are enzymes that catalyze the oxidative deamination of biogenic amines. MAOs are located in the outer membrane of mitochondria and play an important role in the metabolism of neuroactive monoamines such as dopamine, norepinephrine, serotonin, and melatonin in the brain. MAO exists in the form of two isoenzymes, MAO-A and MAO-B. Specifically, epinephrine, norepinephrine, melatonin, and serotonin are mainly metabolized by MAO-A, whereas phenylethylamine and benzylamine are mainly degraded by MAO-B. Both isozymes also have differential cellular localizations in the brain: MAO-A is mainly localized to DAergic axon terminals in the nigrostriatal nucleus, whereas MAO-B is mainly localized to astrocytes and serotonergic neurons. Because of their substrate specificity, selective MAO inhibitors have been designed to inhibit the activity of a specific type of isoenzyme: clorgiline and moclobemide inhibit MAO-A, while selegiline, rasagiline, and safinamide inhibit MAO-B. Most of these MAO-A and MAO-B inhibitors are prescribed in different ways to patients with depression and Parkinson's disease (PD), respectively.

Agnieszka Olejarz-Maciej's doctoral thesis is well-structured and correctly presented. It consists of 5 main chapters written in English, of which the first chapter (Introduction) provides an overview of strategies in the fight against complex diseases, including CNS,

discussion of G protein-coupled receptors as a target of drug action on the example of histamine H_3, H_4 and adenosine A_1, A_{2A} receptors and MAO-B inhibitors. The choice of topics is well-discussed and justified. It allows us to place the experimental work of the Ph.D. student in the context of the achievements of previous research in the search for ligands with multidirectional effects and is based on data from 342 references. In the second chapter (Scope and objectives of the work), Agnieszka Olejarz-Maciej, M.A., described the research plan. In subsection 2.1. entitled "Intrinsic activity of histamine ligands of the human histamine H_3 and H_4 receptor" presents methods for testing the intrinsic activity of a series of histamine ligands of the human H_3 and H_4 receptors using possible secondary messenger pathways. Functional characterization of selected hH_3 receptor ligands was carried out using:
A. cAMP accumulation assay
B.. Ca^{2+} efflux assay
and functional characterization of chosen hH_4 receptor ligands using
A. cAMP accumulation assay
B. β-arrestin recruitment assay using split luciferase complementation
C. β-arrestin recruitment assay using TANGO assay
In subsection 2.2 entitled "Multidirectional activity of human histamine H_3 receptor and adenosine receptor ligands", the following studies were planned and performed:
1. MAO-B inhibition by *h*H_3R ligands
a. initial screening
b. determination of IC_{50} for active compounds
c. determination of MAO-A activity for the most active MAO-B inhibitors
d. structure-activity relationship analysis for MAO-B inhibition
e. structure-activity relationship analysis for dual ligands (*h*H_3R and MAO-B)
2. MAO-B inhibition by A_{1A}R and A_{2A}AR ligands
a. initial screening
b. determination of IC_{50} for active compounds
c. determination of MAO-A activity for the most active MAO-B inhibitors
d. structure-activity relationship analysis for MAO-B and MAO-A inhibition
3. Determination of reversibility of MAO-B inhibition
a. for *h*H_3R ligands
b. for adenosine receptor's ligands
4. Determination of modality of MAO-B inhibition
a. for *h*H_3R ligands
b. for adenosine receptor's ligands
In the third chapter (Research results and discussion), the PhD student presented the results obtained and their detailed analysis. In subsection 3.1. entitled "Intrinsic activity of histamine ligands of the human histamine H_3 and H_4 receptor", for a selected group of ligands of the human histamine H_3 receptor - structurally modified pitolisant analogs (marked as series E) - studies were carried out using the cAMP accumulation test to demonstrate whether the tested compounds were agonists/ antagonists/inverse agonists.
Based on the results obtained, it was concluded that all compounds tested are antagonists and show both high (Ki = 8.8 - 25.2 nM) or significant (K_i = 228.2 - 358 nM) affinity for the H_3 receptor and antagonistic activity in the cAMP accumulation assay (IC_{50}=2.38 to IC_{50}=162 nM). The compounds selected for use in the Ca^{2+} efflux assay were H_3 receptor ligands from the KSK series, in which a linker of varying ω-oxyaliphatic chain length (from 2 to 8 methylene groups) containing hydrophobic substituents such as acetophenone, propiophenone, *p-tert*-butyl- and *p-tert*-amylphenyl was introduced into the 4-piperazine position of the base structure 1-(4-pyridyl)piperazine. The compounds were divided into two groups. The first group included compounds with the same substituents but different chain

lengths. The second group included compounds with the same chain length but different substituents. The reference compound was clobenpropit. Based on the analysis of the results of the affinity for the H_3 receptor and the intrinsic activity measured in the Ca^{2+} efflux test of the derivatives obtained in the KSK series, it was shown that compounds with a linker containing three methylene groups in their structure have the best affinity for the H_3 receptor and intrinsic activity in the Ca^{2+} efflux test. The derivative containing the *p-tert*-butylphenyl residue had the highest affinity for the H_3 receptor, but the high intrinsic activity cannot be attributed to the interaction with the H_3 receptor only - further research is required.

In the next step of the work, the PhD student carried out the functional characterization of selected ligands of the human histamine *h*H_4 receptor based on 1,3,5-triazine-2-amine derivatives using the cAMP accumulation test, i.e. the β-arrestin recruitment test by cleaved luciferase complementation and the β-arrestin recruitment test using the TANGO. The compounds were divided into three groups containing 1,3,5-triazin-2-amine in the 6-position: a) alicyclic substituents b) substituted benzene in the 3- and 4-position c) substituted thiophene. Based on the binding results (K_i) of the tested set of H_4 receptor ligands and the results obtained in the cAMP accumulation test (IC_{50}), it was found that compounds containing phenyl substituents show a similar structure-activity relationship. The situation is different for derivatives containing acyclic substituents and thiophene residues, where differences in the SAR analysis of affinity for the H_4 receptor and intrinsic activity are significant. The observed differences may be due to the presence of substituents that may lead to pathways that reduce the intrinsic activity of the hH4 receptor for all signaling pathways or only for the G protein-dependent pathway. Therefore, in addition to the effect on cAMP, the Ph.D. student tried to study the effect of the above-mentioned compounds on another type of second messenger involved in signaling transduction from the H_4 receptor - β-arrestin.

In the first phase of the study, all compounds were tested in both agonist and antagonist modes in a β-arrestin recruitment assay using cleaved luciferase complementation. Based on the results obtained, compounds that did not show a dose-dependent signal change and those that gave too low a response were excluded from further studies. H_4 receptor ligands - selected to define the detailed internal β-arrestin pathway - have been divided into two groups: partial agonists and inverse agonists and antagonists.

The SAR analysis showed that histamine, used as a reference compound, had the highest agonist activity. Phenylethylene derivatives showed both antagonistic and inverse agonistic activity, whereas compounds with a thiophene ring showed strong, weak antagonistic, or partial agonistic activity depending on the nature and position of the substituent. Derivatives with cycloalkyl, phenyl, and indole substituents showed only antagonistic effects. Based on the analysis of the results obtained in the above-mentioned tests, the Ph.D. student drew a very important conclusion resulting from the discrepancies in the data for the tested group of H_4 receptor ligands. This is the fact that these discrepancies may result from the influence of differences in the structure of ligands on targeted actions or lead to deviations from a specific signaling pathway. In light of the studies presented, it can be concluded that the study of new potential H_4 receptor ligands should include the analysis of their impact on signal transduction pathways. The functional characterization of the above-mentioned H_4 receptor ligands in a β-arrestin recruitment assay using cleaved luciferase complementation was performed at the University of Regensburg. The results presented above lead to the introduction of research based on the recruitment of β-arrestin using the TANGO assay to the Department of Technology and Biotechnology of Drugs of the Faculty of Pharmacy of the Jagiellonian University. To compare the results of the β-arrestin recruitment assays (hH_4R β-arrestin luciferase complementation and the hH_4R TANGO assay), the Ph.D. student selected a series of 1,3,5-triazine-2-amine derivative ligands (characterized by high affinity for the H_4 receptor; K_i<500 nM), containing the following substituents in position 6: straight and branched

aliphatic chains with or without a single double bond, alicyclic rings, aromatic systems, substituted thiophene with different substituents in various positions of the ring and compounds for which different activity has been reported in the literature depending on the test used. In addition, reference compounds (histamine, R-α-methylhistamine, JNJ7777120, thioperamide) were tested. These compounds were tested in both agonist and antagonist mode assays. In the agonist mode of the β-arrestin recruitment assays, the maximum agonist activity achieved by the compounds and the pEC_{50} of the tested agonists were compared. The three compounds that showed no agonist or inverse agonist activity in the β-arrestin luciferase assay (JN-38 - 2-thiophene substituent, TR-7 - p-chlorophenyl substituent, KB-2 - 2-chlorothiophene substituent) also showed no agonist or inverse agonist activity in the TANGO β-arrestin assay. The four compounds that showed agonist activity in the luciferase complementation assay also showed agonist activity in the TANGO assay. (R-(α)-methylhistamine, JNJ7777120, KB-4, KB-30). JNJ77777120 and KB-30 showed higher maximum activity in the luciferase assay, while KB-4 showed higher maximum activity in the Tango assay, R-(α)-methylhistamine showed similar activity in both assays. In the antagonistic mode of the β-arrestin assay, the maximum percentage of antagonistic activity achieved by the compounds and the pIC_{50} of the antagonists were compared. Based on the results obtained, it was shown that: three antagonists (KB-2, TR-7, JN-38) achieved similar percentages of maximal antagonist activity in both tests. Two compounds (JN-13, JN-25) showed higher antagonist activity (>100%) in the Luciferase assay than in the Tango assay (~100%). These two compounds also showed inverse agonist activity in the agonist mode in the luciferase assay. Both KB-4 and KB-30 showed weak antagonistic activity in both assays, while a higher percentage of antagonistic activity was obtained in the Tango assay. Based on the structure-activity relationship analysis - in the antagonist mode of testing - similar results were obtained in both tests (i.e. JN-13 > JN-25; JN-38>KB-2). In addition, both assays were shown to produce similar results in the agonist mode. The luciferase assay was able to detect inverse agonism while the Tango assay was not. In the antagonist mode, the pIC_{50} of the compounds tested were similar, but slightly higher in the Tango assay.

Based on the comparison of both tests, the Ph.D. student concluded that the Tango assay tested a large group of new and reference hH_4R ligands and determined the intrinsic activity of agonist and antagonist compounds. Compared to the hH_4 luciferase assay, the Tango assay takes longer to perform (excluding cell culture and plating). However, the TANGO assay uses less cell media and reagents than the luciferase assay.

The PhD student drew the following conclusions from the SAR analysis: the compound TR-DL-13 with the cyclopenylmethylene moiety had the highest affinity for the hH_4 receptor. This ligand was also the most active antagonist in the TANGO assay. Interesting differences between the affinity for the hH_4 receptor and the intrinsic activity were observed in the group of aromatic derivatives. The presence of chlorine in the ring gave a better affinity for the hH_4 receptor than the presence of a methyl substituent at the same position. The opposite situation occurred in the TANGO test; the methyl substituent gave better intrinsic activity than chlorine. Other interesting observations were reported for the group of thiophene derivatives. The affinity for the hH_4 receptor and the β-arrestin activity of the 2-thiophenyl derivatives were very similar. The presence of the 3-halogen-(2-thiophenyl) moiety provided activity in the agonist mode, whereas the 4 and/or 5 substituted 2-thiophenyl moiety provided activity in the antagonist mode in the β-arrestin assay. Moreover, the 3-thiophenyl moiety provided higher affinity and intrinsic activity than the 2-thiophenyl moiety. Given the results presented, it seems justified to synthesize a larger number of compounds with a 3-thiophenyl moiety containing halides or a methyl substituent in different positions. They may potentially show a better affinity and an interesting intrinsic activity profile.

Subsection 3.2 entitled "Multidirectional activity of human histamine H_3 and adenosine receptor ligands", is the main part of the doctoral thesis and is based on the results described in three experimental articles. These articles were published in international journals (total IF = 11,931). In one of the three articles, the PhD student is the first author and in the other two, she is the second author. Copies of the original articles and handouts have been included with the extensively and thoroughly discussed results. It should be emphasized that, in addition to the above-mentioned articles Olejarz-Maciej, MsC, is the co-author of eleven articles closely related to the topics presented in the doctoral dissertation. To investigate H_3 receptor ligands, that might have additional inhibitory effects on the MAO-B enzyme, the Ph.D. student selected a series of analogs of two lead compounds DL-76 (derivatives of *tert*-butylbenzene) and DL-77 (derivatives of tert-amylbenzene). In the series of derivatives studied, the alkyloxy chain was extended and the pyridine ring was replaced with heterocyclic systems such as pyrrolidine, methylpiperidine dimethylpiperidine derivatives, and azepane. For a series of DL-76 derivatives, based on the structure-activity relationship analysis, the Ph.D. student showed that the greatest MAO-B inhibition was demonstrated by compounds containing a linker with three carbon atoms. As the length of the linker increased, the MAO-B inhibitory activity decreased. The most favorable heterocyclic systems for MAO-B inhibition (for compounds with the shortest, 3-carbon linker) were: pyrrolidine (E-286: IC_{50} = 2.7 nM), 2-methylpiperidine (E-270: IC_{50} = 10.8 nM), azepane (E-265: IC_{50} = 45.3 nM). Analogous analysis for the DL-77 series showed that compounds with 3 carbon atoms containing pyrrolidine (E-289, IC_{50} = 4.5 nM), 2-methylpiperidine (E-293, IC_{50} = 20 nM), 2,6-dimethylpiperidine (E-292, IC_{50} = 22 nM) and azepane (E-296, IC_{50} = 65 nM) showed the highest MAO-B inhibitory activity. Weaker MAO-B inhibition occurred with the addition of 3,3-dimethylpiperidine (E-290, IC_{50} = 417 nM), 3-methylpiperidine (E-294, IC_{50} = 428 nM), and 3,5-dimethylpiperidine (E-291, IC_{50} = 962 nM), To test the selectivity of MAO-B inhibitory potency, the most active MAO-B inhibitors of the DL-76 and DL-77 analogs was tested for possible MAO-A inhibition.

From the DL-76 analog series, all compounds showing MAO-B inhibition were selected, from the DL-77 analog series only compounds with an IC_{50} < 380 nM. Based on the research conducted, Olejarz-Maciej, MsC showed that both DL-76 and DL-77 analogs selectively inhibit MAO-B. Compounds with a carbon linker containing 3 carbon atoms showed the strongest inhibition of MAO-B. A longer carbon chain resulted in weaker MAO-B activity. Replacing piperidine with pyrrolidine, 2-methylpiperidine, 2,6-dimethylpiperidine, azepane, and 3-methylpiperidine resulted in compounds with greater inhibitory potency. Comparison of *tert*-amylbenzene and *tert*-butylbenzene derivatives showed similar MAO-B inhibition properties. In some cases, *tert*-amylbenzene derivatives provided stronger MAO-B inhibition (e.g., piperidine derivatives and 3,3-dimethylpiperidine derivatives with 3 carbon atoms in the linker). Based on the structure-activity relationship analysis of both series of analogs of the lead compounds DL-76 and DL-77, the Ph.D. student identified structural elements that were beneficial for the target ligands showing a dual-directional effect on MAO-B and the *h*H_3 receptor, i.e. a short carbon linker (containing three methylene groups) and a heterocyclic ring such as pyrrolidine, piperidine, 2-methylpiperidine, 3-methylpiperidine, 2,5-dimethyl-piperidine, or azepane.

In the next step of the research, Agnieszka Olejarz-Maciej, MSc, selected several compounds (published or unpublished) from the library of the Department of Technology and Biotechnology of Drugs to determine their dual-directional activity against MAO-B and adenosine receptors. These compounds have been divided into the following derivative subgroups: a) tricyclic xanthines (containing a benzyl, dihydroxybenzylamine, or dopamine substituent and analogs without a third ring) b) theophyllines (containing dopamine in position 8 and an aliphatic or arylaliphatic substituent in position 7), c) theobromine (

containing dopamine in position 8 and an aliphatic or arylaliphatic substituent in position 1), d) hydroxyl-blocked theophyllines and theobromine. Based on the research results obtained, the Ph.D. student drew the following conclusions: a) in the group of tricyclic xanthine derivatives: The most active of the group of tricyclic xanthine derivatives included compounds with one (MZ-1423) or two (JS16006, JS16015, MZ-1504) halogen substituents in the benzyl group. The MAO-B inhibitory activity ranged from IC_{50} = 82.6 nM (JS16015) to IC_{50} = 243 nM (MZ-1504).
The compounds showed selectivity towards the MAO-B isoform (MAO-A IC50 > 1000 nM). Analogous compounds without the third ring did not show MAO-B inhibition (MZ-1476, MZ-1488, MAO-B IC_{50} > 1000 nM).. In the next stage of the research, Agnieszka Olejarz-Maciej, MSc, selected several compounds (published or unpublished) from the library of the Department of Technology and Biotechnology of Drugs to determine their bidirectional activity against MAO-B and adenosine receptors. These compounds have been divided into the following derivative subgroups: a) tricyclic xanthines (containing a benzyl, dihydroxybenzylamine or dopamine substituent and analogs without a third ring) b) theophyllines (containing dopamine in position 8 and an aliphatic or arylaliphatic substituent in position 7), c) theobromine (containing dopamine in position 8 and an aliphatic or arylaliphatic substituent in position 1), d) hydroxyl-blocked theophyllines and theobromine. Based on the research results obtained, the Ph.D. student drew the following conclusions: a) in the group of tricyclic xanthine derivatives: The most active of the group of tricyclic xanthine derivatives included compounds with one (MZ-1423) or two (JS16006, JS16015, MZ-1504) halogen substituents in the benzyl group. The MAO-B inhibitory activity ranged from IC_{50} = 82.6 nM (JS16015) to IC_{50} = 243 nM (MZ-1504).
The compounds showed selectivity towards the MAO-B isoform (MAO-A IC_{50} > 1000 nM). Analogous compounds without the third ring did not show MAO-B inhibition (MZ-1476, MZ-1488, MAO-B IC_{50} > 1000 nM). The absence of the third ring was associated with greater affinity for the adenosine A_2 receptor (MZ-1476: K_i = 283 nM, MZ-1488: K_i = 148 nM). The change from a mono- or dihalosubstituted benzyl moiety to a dihydroxybenzyl substituent (MZ-1470) resulted in lower MAO-B inhibitory activity (IC_{50} > 1000 nM) as well as no affinity for adenosine receptors Dihydroxybenzylamine to dopamine substitution (KD-393 and KD-452)) showed some MAO-B inhibitory activity (KD-452: IC_{50} = 97.2 nM, KD-393: IC_{50} = 155 nM), but the compounds did not reach maximum inhibitory activity. In group b) theophylline derivatives. All derivatives showed partial inhibitory activity against MAO-B. Among the aliphatic theophylline derivatives, compounds with methyl, ethyl, butyl, pentyl, and hexyl substituents in position 7 showed similar activity against MAO-B (IC_{50} = 41.5 - 76.4 nM; 49-62% of maximum inhibitory activity). The weakest activity was demonstrated by the compound with the propyl substituent in (KD-403: IC_{50} = 152 nM, 51%). The elongation of the aliphatic chain resulted in a decrease in affinity for the A_2 receptor (from KD-444 K_i = 370 nM (for a linker containing one carbon atom) to KD-445 K_i > 10000 (for a linker containing 6 carbon atoms). The situation was different for the A_{1A} receptor, where the affinity increased from K_i = 1980 nM for the KD-444 derivative to K_i = 304 nM and K_i = 191 nM for compounds containing linkers with 4 and 5 carbon atoms (KD-437 and KD-459). In group c) of theobromine derivatives, the elongation of the carbon chain caused an increase in the maximum inhibition (from methyl to hexyl substituent: DS-14: 71%, DS-13: 87%, DS-16: 91%, DS-22: 97%), further chain elongation to heptyl and octyl did not cause any changes in the inhibitory potency of the tested compounds (DS-18: 100%, DS-20: 97%). All arylalkyl theobromine derivatives achieved more than 93% of the maximum MAO-B inhibition. For compounds with an unsubstituted aromatic ring, lengthening the aliphatic linker from one (DS-6: IC_{50} = 121 nM) to three (DS-26: IC_{50} = 91.8 nM) carbon atoms only slightly increased MAO-B activity. The introduction of one chlorine (DS-7: $IC5_0$ = 111 nM) or one bromine

(DS-24: IC_{50} = 112 nM) into the benzene ring did not change the inhibitory potency against MAO-B compared to the unsubstituted ring. In contrast, the presence of fluorine in the compound (DS-28: IC_{50} = 37.3 nM) increased the inhibitory potency against MAO-B. The introduction of two chlorine atoms at the 2,4 positions resulted in a slightly higher inhibitory potency (Tb-1: IC_{50} = 2.61 nM) than their presence at the 3,4 positions (IC_{50} = 13.4 nM). In the group of derivatives d) theophylline and theobromine with blocked hydroxyl groups. The blockade of hydroxyl groups in theophylline and theobromine derivatives resulted in a decrease in MAO inhibitory activity but did not decrease the affinity for A_{2A} receptors for most compounds. Compounds with interesting MAO-B inhibitory properties were tested against MAO-A to check the selectivity of the inhibitors (at one, two, or three concentrations). The group of alkylaromatic theobromine derivatives that were most active in the MAO-B test showed some MAO-A activity and were therefore selected for dose-dependent experiments. Several theophylline derivatives were also selected for MAO-A tests. Theophylline and theobromine derivatives with a dopamine moiety were able to inhibit the MAO-A isoenzyme. In addition, none of the compounds tested showed complete MAO-A inhibition. The highest percentage of maximum inhibition was achieved by compounds DS-7, and DS-24 (80%). All theophylline derivatives tested in a dose-dependent manner for both isoenzymes showed lower IC_{50} for MAO-B but achieved a higher percentage of maximal MAO-A inhibition. However, in the case of theobromine derivatives, the compounds tested in a dose-dependent manner for both isoenzymes showed better IC50 and achieved a higher percentage of maximum MAO-B inhibition.

In the final step of the work, the Ph.D. student investigated the reversibility of inhibition and the modality of MAO-B inhibition for ligands of the human histamine H_3 receptor and ligands of the A1 and A_{2A} adenosine receptors.

To study the reversibility of MAO-B inhibition, the Ph.D. student selected the most active MAO-B inhibitors from the group of DL-76 and DL-77 analogs with dual-directional action (hH_3, MAO-B). Tricyclic xanthine derivatives: MZ-1423, JS16015, JS16006, and MZ-1504 and theobromine derivatives: DS-9, Tb-1, and DS-6 were selected for the study of the reversibility of MAO-B inhibition by adenosine receptor ligands. All selected compounds were tested with two reference inhibitors: the reversible safinamide and the irreversible rasagiline. Based on the results obtained, it was shown that all the tested derivatives exhibited reversible inhibition of MAO-B. After confirming the reversibility of the tested compounds, 3 compounds and safinamide from the group of derivatives (hH_3, MAO-B) and 2 compounds from the group of adenosine receptor ligands were selected for kinetic tests (MZ-1504, Tb-1). For all compounds tested, a mixed mode of inhibition was confirmed, with greater affinity for the free enzyme than for the enzyme-substrate complex. A similar modality, under the same assay conditions, was observed for the reference reversible inhibitor - safinamide.

Part 4 of the dissertation thesis submitted for review contains a summary of all the research carried out. Part 5 entitled "Materials and methods" contains a description of the methods used and the materials used to carry out the planned research.

The undoubted achievement of the doctoral thesis submitted for review is the investigation of the intrinsic activity of a series of histamine ligands of human H_3 and H_4 receptors using the pathways of possible secondary messengers. The concept of intrinsic efficacy has been well established in pharmacology for seventy years, but recent data have shown that multiple ligands can differentially activate signaling pathways mediated by a single G-protein-coupled receptor in ways that challenge the traditional definition of intrinsic efficacy. One of the terms used to describe this phenomenon is functional selectivity. At the extreme, functionally selective ligands can be both agonists and antagonists for different functions mediated by the same receptor. In addition to the heuristically interesting nature of functional selectivity, there are clear implications for drug discovery as this mechanism

increases the ability to select or design new ligands that differentially activate only a specific subunit of a single receptor, thereby optimizing therapeutic effect.
The search for drugs with multidirectional effects, as is in the case of this Ph.D. thesis, is of great importance in multifactorial CNS diseases such as depression, schizophrenia, Parkinson's disease (PD), and Alzheimer's disease (AD). If a single drug molecule can be designed to interact with multiple targets simultaneously, neurodegenerative diseases can be treated with greater efficacy, lower toxicity, and fewer drug-drug interactions. The in vitro activity results presented in this doctoral dissertation, undoubtedly provide important insights into the structure-activity relationship, which should contribute to the design of new functionally selective multi-target ligands.

In conclusion, the main objectives of the thesis have been met. The results are well presented and their interpretation is at a high scientific level. All experiments have been well planned and carried out, and the measurement techniques and methods have been correctly applied.

However, in addition to this highly substantive assessment of the achievements presented, I also have a few critical remarks. Minor inaccuracies, omissions, and typographical errors do not significantly affect the overall assessment of the work. However, the title of the thesis "Multidirectional action of new ligands of histamine and adenosine receptors" is misleading. The research presented only looks at H_3 and H_4 histamine receptor ligands, not the other two. Similarly, for adenosine receptors - only the A_1 and A_{2A} receptor ligands were tested and not all known ones - what was the reason for such a generalization of the topic of the doctoral dissertation?

In my opinion, the reviewed doctoral thesis fulfills all the requirements for doctoral theses laid down in Art. 13 section 1 of the Act of 14 March 2003 on academic degrees and scientific titles as well as degrees and titles in the field of art (Journal of Laws of 2017, item 1789, as amended), and demonstrates the Ph.D. student's ability to think critically and work in a scientific team in the field of biochemistry and pharmacology. Therefore, I am appealing to the Disciplinary Council of Pharmaceutical Sciences at the Jagiellonian University in Krakow to admit Agnieszka Olejarz-Maciej, MsC to further stages of the proceedings regarding the award of a doctoral degree in the field of medical and health sciences, in the discipline of pharmaceutical sciences.

Given the enormous amount of work carried out by the Ph.D. student and the research results obtained, which may contribute to the design of new functionally selective ligands with multidirectional activity, I am requesting that the reviewed doctoral dissertation be awarded (reasons attached).

Signed by /
Podpisano przez:

Krzysztof Włodzimierz
Walczyński
Uniwersytet
Medyczny w Łodzi

Date / Data:
2023-09-12 17:31