FUNCTIONALIZED CELLULOSE AS A MATRIX FOR THE SYNTHESIS OF LIBRARY OF MOLECULAR RECEPTORS USEFUL FOR SCREENING OF COMPOUNDS WITH ANTI-HISTAMINE ACTIVITY

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Abstract

The library of molecular receptors was formed by self-organization of N-heptanoylated dipeptides anchored in the regular fashion via aminophenyla-mino-1,3,5-triazine linker to the surface of cellulose membrane. SPOT method was used for the synthesis of peptide library. As C-terminal amino acids of peptide fragments were attached: Ala, Pro and Phe, while as a N-terminal amino acids were applied all natural amino acids. DMT/NMM/TosO- was selected as a coupling reagent for synthesis of library of N-heptanoylated dipeptides. These constructs were used as a tool for distinguishing pharmaceutically active compounds acting on histamine receptors. In the studies as active compounds were tested: Doxylamine and Difenhydramine with histamine agonist activity, Ranitidine and Cimetidine with antagonist activity as well as Histamine – natural ligand. The binding of colourless ligands was monitored by staining with Brilliant Black used as reporter dye and quantitative colour measurement was performed in 256 grade gray scale by using Image-Quant software. Substantial differences in the ability of interactions of agonists and antagonists with bounding pockets were observed with selected molecular receptors. From 60 elements library of molecular receptors were selected 12, which were able to distinguish between agonists or antagonists. It has been found that even small changes (Leu residue vs Val residue) in the structure of molecular receptor influenced specificity of agonist or antagonist binding.

***Keywords:** molecular receptors, immobilized peptides, binding pocket, agonist/antagonist, histamine receptors*

Introduction

Cellulose is a biopolymer composed of β D-glu-copyranose residues bonded with 1,4 glycosidic bonds. Its characteristic feature is the equatorial arrangement of C2, C3 secondary hydroxyl groups and the primary C6-hydroxylmethyl group. The CH_2OH group is in trans-gauche position with respect to O5-C5 and C4-C-5 bonds. The result of this arrangement is the regular structure of cellulose resulting from the high content of crystalline phase and small content of the amorphous one.

Cellulose has four allomorphic forms: I (Iα, Iβ), II, III, IV. Cellulose I and cellulose II form the crystalline phase. In both cases, the presence of hydrogen bonds between the O3-H ---- O5 within the chain causes the formation of rigid linear chains. The differences between both allomorphic forms of cellulose depend on the antiparallel arrangement of chains of cellulose II and on the occurrence of additional hydrogen bonds. For cellulose I, a hydrogen bond is between O6-H ---- O3 and in the case of cellulose II this bond is formed between O6-H ---- O2 [1]. Another great advantage of cellulose is the precisely defined reactivity of hydroxyl groups in each subsequent chain. It is generally assumed that primary hydroxyl groups are about 25 times more reactive than secondary ones [2]. It is also important that cellulose is readily available, both as the most abundant material produced by plants and microbes. Different origin of cellulose is causing this material available in different forms, ranging from nano-, micro- to biocellulose [3-5]. Regardless of the origin of cellulose, the procedures for reducing amorphous areas by hydrolysis are crucial to the properties of the biopolymer. Presented facts make cellulose and its derivatives widely applicable. The most sophisticated are application on chiral stationary phases [6], diaphragms used in filtration [7], membranes used in SPOT technology [8] up to the application as components of drugs formulation and drug delivery systems [9,10].

Recently, it has been shown that cellulose can be used as a support for anchoring on its surface *N*-lipidated peptides, which are forming binding cavities mimicking natural receptors and/or enzymes [11,12]. The key factor to achieve enzymatic activity or ligand binding capacity is the dynamic adaptation of peptide chains forming molecular receptors, by induced conformational changes, to the shape of the bounded ligand [13]. Two factors are crucial for efficient ligand binding: the space between immobilized peptides and the presence of elements offering most of weak interactions including π-acceptor, π-donor, van der Waals forces, hydrophobic interactions, ionic bonds and hydrogen bonds [14]. The interactions between binding pocket and docked ligand are highly selective and binding cavities are able to differentiate ligands recognizing their size, shape, charge distribution, chirality and polarity. High selectivity of interactions between molecular receptor and ligand is very important because the biological activity can be determined by even small changes in the ligand structure. The binding strength of ligands through the molecular receptors is the outcome of all interactions between the host and the guest and depends on both the structures of the ligand and the molecular receptor. The process of binding is reversible due to the nature of weak interactions between ligands and the binding pockets (FIG. 1).

Additionally, it has been found that the mechanism of binding is competitive and therefore the described process is mimicking the interactions involving natural receptors. This inspired us to use molecular receptors as tools for the testing of pharmacologically active compounds. The goal of these studies was to test whether a library of molecular receptors formed by self-organization of peptides immobilized on cellulose can mimic a histamine receptor and thus be useful in evaluation antihistamine active compounds diversifying their mode of action. It has been expected that it would be possible to select molecular receptors selectively interacting with agonists and antagonists.
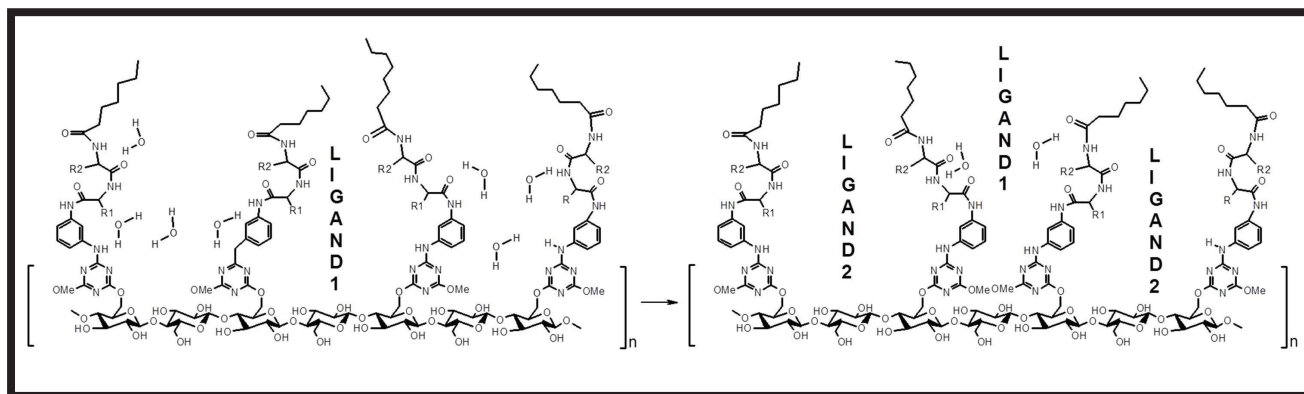
FIG. 1. Competitive, reversible mechanism of docking ligands into binding pockets.

Materials and Methods

General information

During the synthesis were used: tetrahydrofuran (THF), dichlormethan (DCM), methanol (MeOH) (POCH, 99.8%); N,N-dimethylformamid, (DMF) (J.T.Baker, 99.9%); 1-methyl-2-pyrrolidinone, (NMP) (Acros Organics, >99.5%); Piperidine, (SAFC, >99%); 4-methylmorpholine, (NMM) (Alfa Aesar, 99%); all Fmoc-protected amino acids (GL Biochem, >98.5%); 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium 4-toluenesulfonate (DMT/NMM/ TosO⁻) prepared according optimized procedure [15], Histamine, Diphenhydramine, Doxylamine, Cimetidine, Ranitidine, Brilliant Black BN (tetrasodium (6Z)-4-acetamido-5-oxo-6-[[7-sulfonato-4-(4-sulfonatophenyl)azo-1-naphthyl]hydrazono]naphthalene-1,7-disulfonate) (Sigma Aldrich, >98%).

Immobilization of 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT): 6 sheets (10 cm x 15 cm) of Whatman-7 filter paper were immersed in 1M NaOH (40 ml) and gently shaken for 15 min. An excess of solution was removed, then the wet paper sheets were soaked in suspension of finely grounded sodium bicarbonate in 1M solution of 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) (6.8 g) in THF (40 ml) and again gently shaken for 60 min at room temperature (rt). Then cellulose sheets were washed with THF (2 x 30 ml), acetone (2 x 30 ml), acetone:H$_2$O 1:1 (2 x 40 ml), acetone (2 x 40 ml) and with DCM (30 ml), than left to remove the remaining solvent and dried in a vacuum desiccator. Elemental analysis for cellulose was found: %N 0.00-0.05 and %Cl 0.01-0.05, after DCMT immobilization it was found: %N 3.64 and %Cl 2.66. Loading of the cellulose sheets with triazine was calculated from elemental analysis data. Surface loading calculated according to nitrogen content was $31.9 \cdot 10^{-6}$ mol (N)/cm^2; which was equivalent to NL^s = $10.6 \cdot 10^{-6}$ mol (triazine)/cm^2 and CIL^s = $9.2 \cdot 10^{-6}$ mol (Cl)/cm^2.

Immobilization of m-phenylenediamine: The cellulose sheets functionalized with DCMT were immersed in 1 M solution of *m*-phenylenediamine (4.3 g) in THF (40 ml) and gently shaken for 24 h at rt. Then sheets were washed successively with THF (2 x 20 ml), DMF (2 x 20 ml), again THF (2 x 20 ml) and dried in desiccator.

SPOT synthesis of N-hepatnoyled peptides on the cellulose: The peptides were performed on automatic synthesizer ResPep SL by Intavis using SPOT synthesis. The 0.56 M solutions of all Fmoc-protected natural amino acids were prepared in NMP. As a coupling reagent was used a 0.5 M solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium 4-toluenesulfonate (DMT/NMM/TosO⁻) in DMF. As a base 2 M NMM in DMF was used. To remove Fmoc protecting group 25% piperidine in DMF was used.

The first synthetic cycle included: Preactivation: 6 min, for each spot: 0.15 µl solution of coupling reagent; 0.073 µl solution of NMM; 0.17 µl amino acid (Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Phe-OH); 0.003 µl NMP; Coupling: 2 x 3 min; for each coupling was used 0.198 µl of preactivated solution. *The second synthetic cycle included*: Fmoc-group deprotection (2 x 10 min, dispense volume: 500 µl); Preactivation: 6 min, for each spot: 0.15 µl solution of coupling reagent; 0.073 µl solution of NMM; 0.17 µl amino acid (all natural amino acids); 0.003 µl NMP; Coupling: 2 x 3 min; for each coupling is used 0.198 µl of preactivated solution. *The third synthetic cycle included*: Fmoc-group deprotection (2 x 10 min, dispense volume: 500 µl); Coupling: 1 x 7.5 min; for each spot were used: 0.3 µl NMP and 0.15 µl of 0.25 M solution of triazine esters of heptanoic acid in DMF:NMP (1:1).

Deprotection of the side chains of dipeptides: Modified cellulose membranes were treated with mixture consisting of 50% (v/v) trifluoroacetic acid in DCM (200 ml) with 3% (v/v) water and 2% (v/v) trisopropylsilane for 5 h. Then the membranes were washed with DCM (2 x 100 ml), EtOH (2 x 100 ml) and dried in a vacuum desiccator.

Buffering of cellulose membranes: The modified cellulose membrane were buffered with phosphate buffer pH 7.0 (200 ml) for 30 min. Then, the celluloses were washed with water (2 x 200 ml) and with mixture MeOH : H$_2$O (1:1) and dried to constant weight in the vacuum dessicator. During the synthesis two identical libraries were synthesized on each functionalized sheet, after splitting each sheet for two parts, one of them was treated with active substance and then with Brilliant Black, the second one was reared with the reporter dye only.

The docking of active substances to the binding pockets of library N-heptanoyled peptides immobilized on the cellulose: The cellulose sheets were treated with 10 ml 0.002 M solution of active substances: Histamine, Diphenhydramine, Doxylamine, Cimetidine, Ranitidine. After 30 min the excess of the solution was removed and the sheets were washed with MeOH (5 x 100 ml). After drying to the constant weight, the cellulose sheets were treated with 1.25 mM solution of the Brillant Black in MeOH for 30 min. Then the solution was removed and sheets were washed with MeOH (8 x 100 ml). Cellulose membranes after staining were dried in vacuum desiccator, scanned and processed using Image-Quant program. Ability of molecular receptors to interact with colourless active compounds was calculated as difference in intensity of coloration caused by docking reporter dye and intensity of coloration after treatment with colourless ligand and subsequently with reporter dye. In this way, for each spot average value of "gray" coloration corresponding to interaction between binding pocket of molecular receptor and antihistamine ligand was determined.
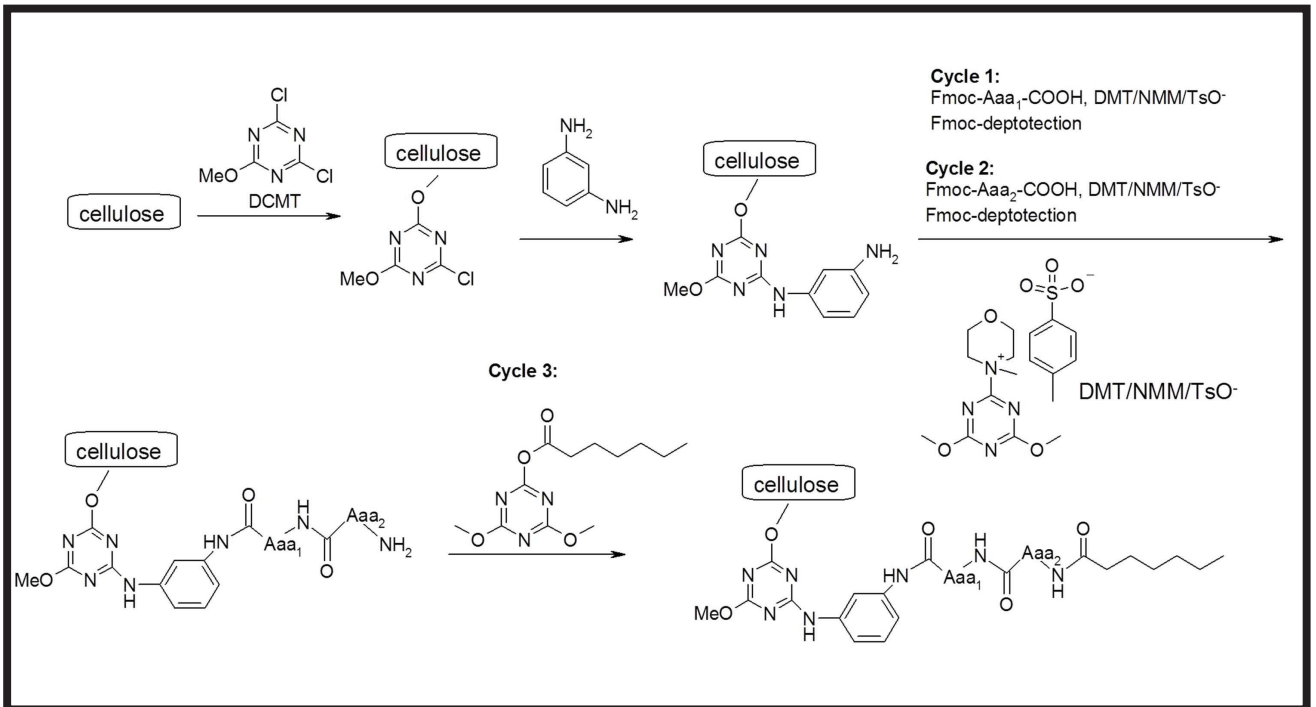
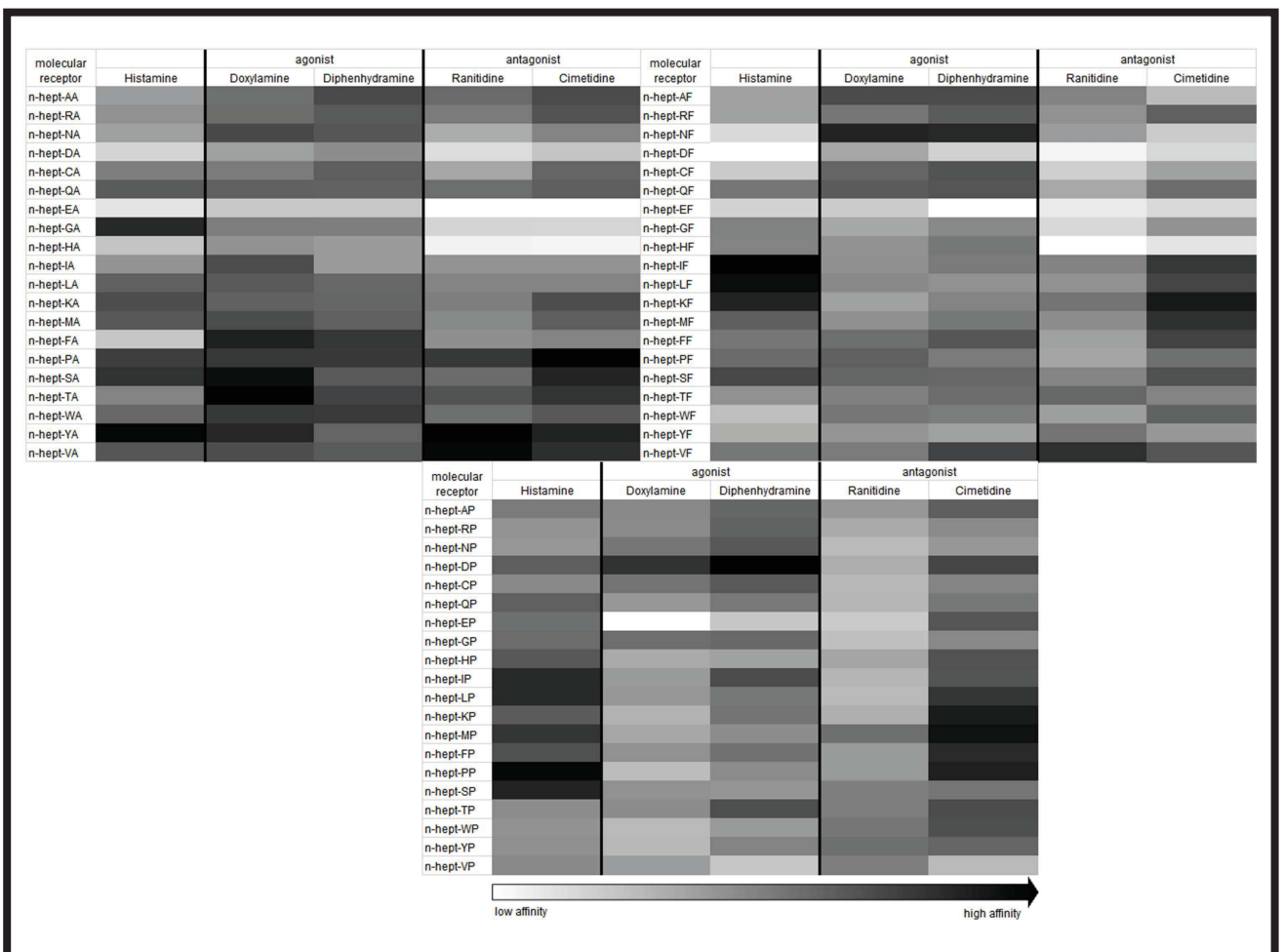**FIG. 2. Synthetic pathway for preparation of *N*-heptanoylated dipeptides immobilized on cellulose.**

FIG. 3. The map of intensity of bounding of tested compounds by cavities of library of molecular receptors immobilized on cellulose, dark coloration - strong molecular complex, light coloration - weak molecular complex.
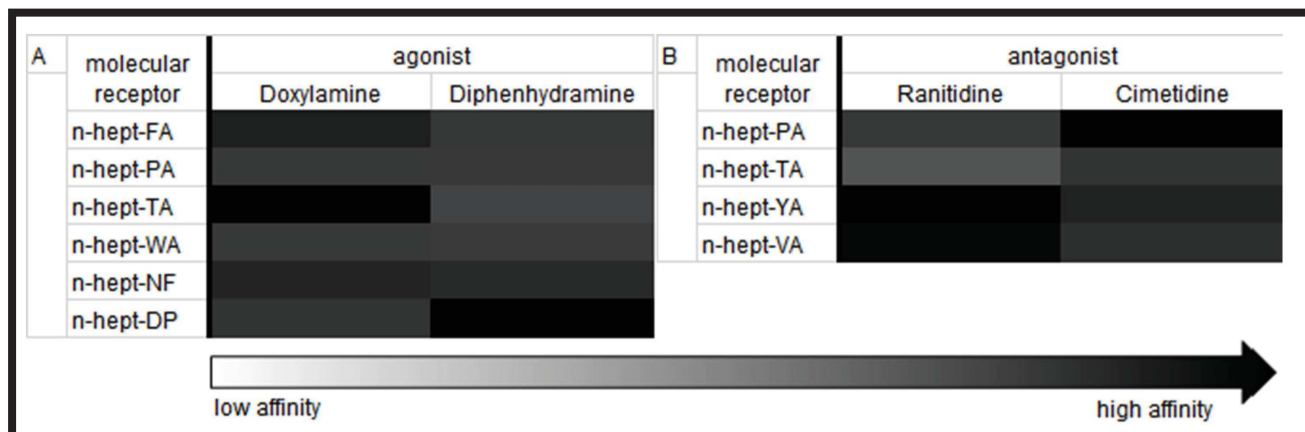
FIG. 4. The map of intensity of bounding of tested compounds by selected molecular receptors giving strong molecular complexes.

Results and Discussions

In the study all natural amino acids were used as substrates for the synthesis of a library of molecular receptors. A randomized approach to design the peptide fragments was preferred due to complexity of the search for molecular receptors capable of differentiating affinity of agonists and antagonists of histamine receptors. The molecular receptors were prepared using a stepwise process involving functionalization of cellulose with a 2,4-dichloro-6-methoxy-1,3,5-triazine derivative followed by reaction with *m*-phenylenediamine, coupling with *N*-Fmoc protected Ala, Pro or Trp, deprotection of the *N*-terminus, coupling with all Fmoc protected proteinogenic amino acids, and finally acylation with *n*-heptanoic acid (FIG. 2). Synthesis of *N*-heptanoylated dipeptides attached on the surface of the modified cellulose membrane was done by using the automated SPOT technique. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium 4-toluenesulfonate (DMT/NMM/TosO⁻), a coupling reagent with well documented efficiency in peptide synthesis, was applied [15]. Doxylamine and Difenhydramine with histamine agonist activity, Ranitidine and Cimetidine with antagonist activity as well as Histamine – the natural ligand, were used in the studies involving molecular receptors acting as a histamine receptors model.

Using a competitive mechanism of ligand binding to cavities of molecular receptors, it was possible to visualize molecular complexes formed with colourless ligands applying the procedure with reporter dye [16].

The ability of molecular receptors to interact with colourless active compounds was calculated as a difference in intensity of coloration caused by docking reporter dye only and intensity of coloration after treatment the docked colourless ligand with the reporter dye. In this way, bigger difference in the coloration corresponded to stronger binding of ligand by receptor. This was finally presented on the interactions map by more dark "gray" coloration. Brilliant Black was used as the reporter dye. This dye was selected because it gave fairly uniform coloration of most receptors, weakly depended on the structure of the binding pocket. The observed intensity of coloration of spots corresponded to the stability and the strength of the molecular complexes formed between bounding cavities of molecular receptor and docked ligand. FIG. 3 shows the interaction map of the molecular receptor library with tested anti-histamine active compounds. It has been found that even a randomized library of *N*-heptanoylated dipeptides evidenced a significantly different susceptibility to docked pharmaceutically active compounds.

The ten molecular receptors forming strong molecular complexes with both agonists and antagonists were selected and presented in FIG. 4.

Structural analysis of selected molecular receptors has indicated that in 8 out of 10 cases, the *C*-terminal amino acid of the peptide was alanine. With regard to the structure of *N*-terminal amino acid, there was a considerable variation as follow. Both aromatic amino acids, as well as hydrophobic aliphatic amino acid, and even amino acids with hydroxyl functions in the side chains were identified. Taking into account that all docked derivatives are amines, it has to be underlined that only in one case the molecular receptors contained amino acid with a carboxylic function in the side chain (Asp-Pro). This result indicates that a rather mutual adjustment between guest-host by using hydrophobic interaction and/or π-interactions is critical for the formation of stable molecular complexes. Ionic bond between the carboxyl group in the side chain of the dipeptide and the amine function of the docked ligand appear less crucial. Most important for this study was to search for molecular receptor structures for which the ability to interact would be different for agonists and antagonists, that is, depended on the biological activity of ligands. It has been found that even in the model library of molecular receptors it was possible to find such structures (FIG. 5). The observed differences in stability of molecular complexes formed with agonists and antagonists were statistically significant.

From the pool of 60 molecular receptors, 12 dipeptides were capable of distinguishing between agonists and antagonists, based on the formation of molecular complexes of substantially different stabilities. For molecular receptors with a high affinity for antagonists, the predominant residue at the *C*-terminal position of peptides was proline, while *N*-terminal amino acids residue were hydrophobic. For a pool of molecular receptors with higher affinity for agonists, in most cases at the *C*-terminal alanine residue was present. Particularly noteworthy are four molecular receptors: LeuAla, ValAla, TrpAla, TrpPro. In the case of receptor containing LeuAla there was observed higher affinity toward agonist, whilst changing leucine residue for valine (ValAla) altered the specificity toward antagonist. The similar situation was observed for TrpAla and TrpPro, exchanging *C*-terminal amino acid residues gave the receptor with different affinity against agonist or antagonist.
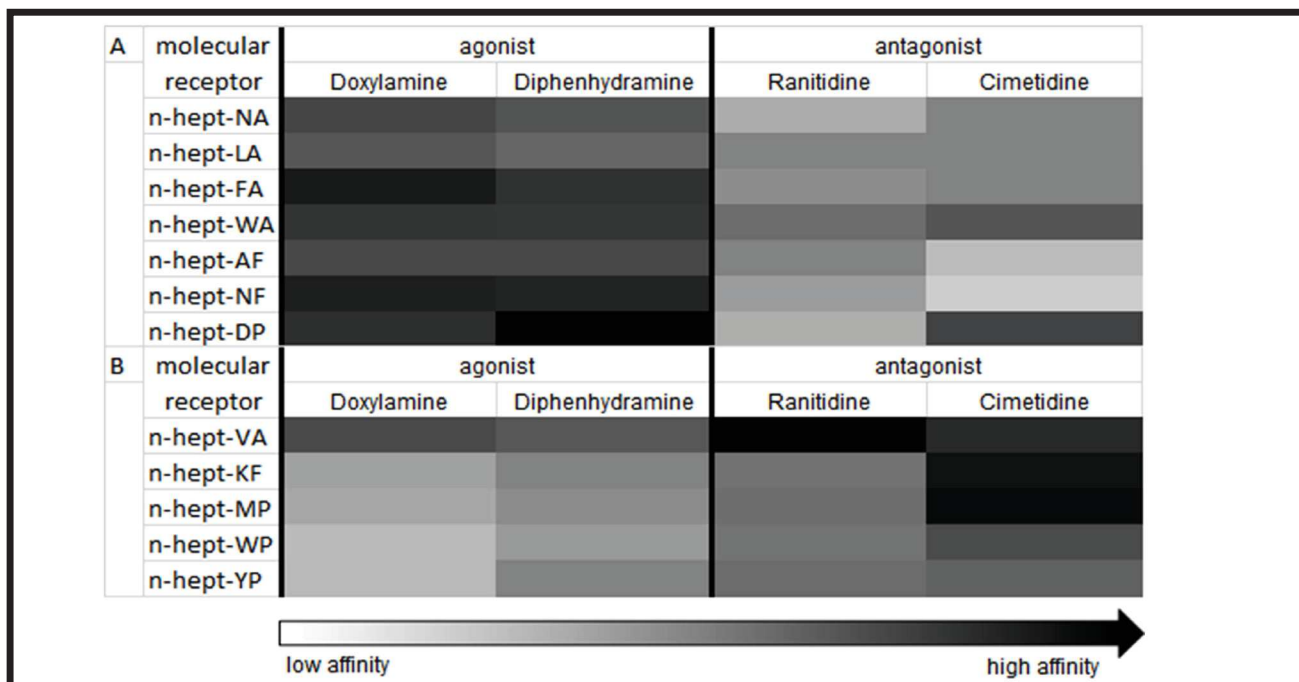
FIG. 5. The map of intensity of bounding of tested compounds by molecular receptors distinguishing agonists from antagonists.

Conclusions

These studies revealed that the library of molecular receptors is recognizing and differentiating agonistic/antagonistic profile of antihistamine active compounds. Even not understanding of complex relations between the structure of the molecular receptor and structure of the pharmacologically active substance, this should allow the construction of a new research tool useful as a platform for screening of new antihistamine compounds. Works on the synthesis of a second generation molecular receptors library based on selected molecular receptor structures and constitutive histamine H1-H4 receptor fragments are underway.

Acknowledgments

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