



**BUDAPESTI MŰSZAKI ÉS GAZDASÁGTUDOMÁNYI EGYETEM
VEGYÉSZMÉRNÖKI ÉS BIOMÉRNÖKI KAR
OLÁH GYÖRGY DOKTORI ISKOLA**

**FRAGMENT-BASED APPROACHES IN GPCR-TARGETED
DRUG DISCOVERY**

Thesis booklet

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2018

1. INTRODUCTION

In my PhD work I aimed the development of ligand-, and structure-based virtual screening methodologies for the design of aminergic GPCR ligands. The prospective screening of our in-house fragment library by a physicochemical property-based filtering and consensus scoring based docking methods led to the identification of new chemotypes such as spiro[indoline-3,3'-pyrrolidines], and spiro[pyrrolidine-3,3'-oxindoles] as 5-HT₆R and 5-HT₇R ligands, respectively. The usefulness of the consensus scoring methodology was also demonstrated by the design of 5-HT_{2B}R antagonists.

2. BACKGROUND

2.1. Fragment-based Drug Discovery

The first reported successful FBDD-driven study was published in 1996 presenting a SAR (structure-activity relationship analysis) by NMR approach¹, followed by several success stories of companies such as Astex, Vernalis and Sunesis around the 2000s. FBDD is a well-appreciated strategy resulting in a significant number of chemical probes, clinical candidates and marketed drugs, as for example: Verubecestat, Vemurafenib and Venetoclax.

Fragment screening hits can be optimized to lead compounds with improved affinities and selectivities across a panel of targets by pharmacophore modelling, structure-activity relationship analyses, structure-based modelling, and by following different fragment-optimization strategies, such as fragment linking, fragment merging, fragment growing.

2.2. G-Protein coupled receptors

Receptors are microswitches/transmitters of chemical communication between cells. Endogenous ligands (neurotransmitters, hormones, cytokines, mediators, intracellular messengers, etc.) of receptors are specifically enabled at the appropriate location and concentrations, and bind to the orthosteric pockets of the receptor macromolecules. All further secondary sites are considered as allosteric pockets. Receptors are capable of transforming the binding event into signal transmission towards other proteins and messengers. G-protein-coupled receptors (GPCR) are localized in the cell-membrane, effecting signalization through G-protein and β -arrestin. GPCR's are expressed in the periphery and the central-nervous system, functionally coupling to a heterotrimeric (α , β , γ) G-protein.

The approx. 40 % of marketed drugs target GPCR's, thus representing their importance in medicine. Treatment of CNS-related symptoms (Alzheimer's Disease,

¹ Shuker SB, Hajduk PJ, Meadows RP, Fesik SW. Discovering high-affinity ligands for proteins: SAR by NMR. *Science*. 1996;274(5292):1531-1534. doi:10.1126/science.274.5292.1531.

schizophrenia, depression, cognitive deficit, anxiety, etc.), and other important diseases such as hypertension, diabetes, sepsis, obesity, cancer, etc. are all covered by the indications of GPCR targets.

2.3. Serotonin receptor subtype-6 (5-HT₆R)

The 5-HT₆R is considered as a current and promising drug target for the treatment of CNS-related indications², such as cognitive, learning and memory deficits related to Alzheimer's disease, Parkinson's disease and schizophrenia. Other CNS related indications such as analgesia, obesity, anti-drug-abuse, sleep-wake regulation, self-transcendence, executive cognition, and major depressive disorder have also been suggested.

Pharmacophore based approaches on 5-HT₆R antagonists are typically focused on the canonical pharmacophore model³ (**Figure 1**), which is defined as having two hydrophobic rings/ring systems (HYD₁ and HYD₂) connected by a hydrogen bond acceptor (e.g. sulfonyl, sulfonamide linker). Optionally, a positively ionizable group (connected to HYD₁) offers interactions with the conserved Asp^{3.32} aspartate residue of aminergic GPCR's as a key factor of aminergic 7TM-receptor activation⁴. A further pharmacophore feature might contain an additional intramolecular hydrogen bond donor moiety further stabilizing the binding conformation of the ligands⁵. Selectivity against other aminergic GPCR's was shown⁶ to be accessible through omitting the positively ionizable group in the 5-HT₆R antagonists.

² Kelemen ÁA, Mordalski S, Bojarski AJ, Keserü GM. Computational Modeling of Drugs for Alzheimer's Disease: Design of Serotonin 5-HT₆ Antagonists. In: Vol 132. ; 2018:419-461.

³ Holenz J, Mercè R, Diaz JL, et al. Medicinal Chemistry Driven Approaches Toward Novel and Selective Serotonin 5-HT₆ Receptor Ligands. 2004.

⁴ Deupi X, Standfuss J, Schertler G. Conserved activation pathways in G-protein-coupled receptors. *Biochem Soc Trans.* 2012;40(2):383-388.

⁵ López-Rodríguez ML, Benhamú B, de la Fuente T, Sanz A, Pardo L, Campillo M. A three-dimensional pharmacophore model for 5-hydroxytryptamine₆ (5-HT₆) receptor antagonists. *J Med Chem.* 2005;48(13):4216-4219.

⁶ Harris RN, Stabler RS, Repke DB, et al. Highly potent, non-basic 5-HT₆ ligands. Site mutagenesis evidence for a second binding mode at 5-HT₆ for antagonism. *Bioorg Med Chem Lett.* 2010;20(11):3436-3440.

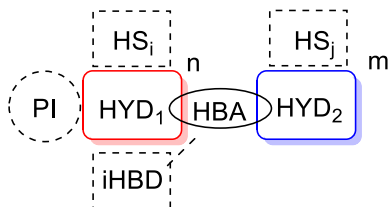


Figure 1. General pharmacophore model of 5-HT₆R antagonists. PI: positive ionisable portion; HYD_{1,2}: hydrophobic sites; iHBD: intramolecular hydrogen bond donor site; HBA: hydrogen bond acceptor feature; HS_{i,j}: substituents on the hydrophobic sites.

The following four 5-HT₆R antagonists reached clinics as drug candidates (**Figure 2**): Intepirdine/RVT-101/SB-742457 **1** (phase 3 - failed; GSK, Axovant), Lu-AE58054/idalopirdine/SGS-518 **2** (phase 3 - failed; Lundbeck), SUVN-502 **3** (phase 2; Seven LifeSciences), PF-0521377/SAM-760 – undisclosed structure (phase 2; Pfizer).

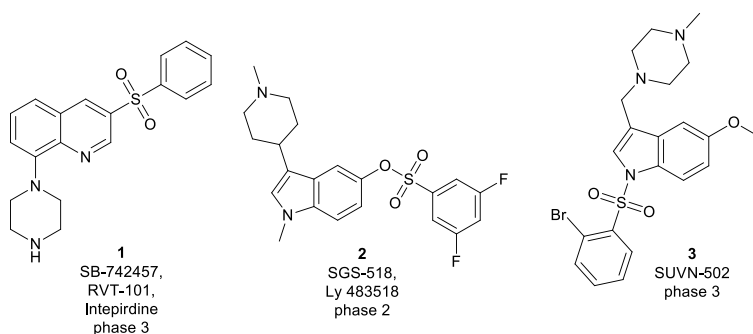


Figure 2 – Structures of the most prominent 5-HT₆R drug candidates reached clinical phases

2.4. Serotonin receptor subtype-7 (5-HT₇R)

The 5-HT₇R receptor plays role in the regulation of body temperature, sleep-wake rhythm, circadian rhythm, and mood. Thus, this receptor has become rapidly an important target for several important CNS-related indications⁷ such as depression sleep disorders, stress, anxiety, learning/memory deficits, schizophrenia-like cognitive

⁷ Nikiforuk A. Targeting the serotonin 5-HT₇receptor in the search for treatments for CNS disorders: Rationale and progress to date. *CNS Drugs*. 2015;29(4):265-275.

deficits, epilepsy, migraine, autism spectrum disorders, and Rett syndrome.

To date one drug candidate with the pyrazolo[3,4-d]azepine core (JNJ-18308683, **4**) has reached the clinics (phase 2 currently recruiting), and a number of investigational compounds has been proved to be selective antagonists (e.g. **5**, **6**) (**Figure 3**).

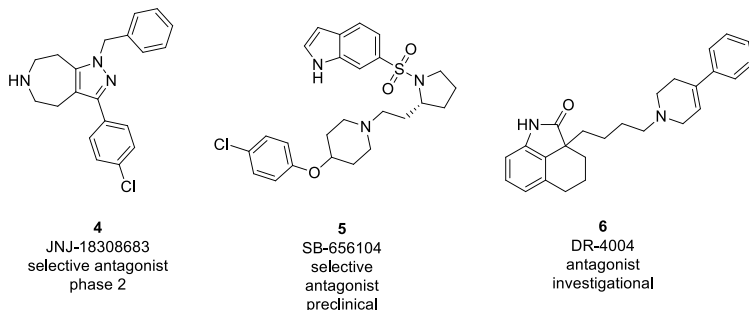


Figure 3 – Structures of the most prominent 5-HT₇R selective antagonists

The pharmacophore model⁸ of 5-HT₇R (**Figure 4**) defines one aromatic structural part (stacking with Phe^{3,28} and Tyr^{7,43}), and two further hydrophobic regions HYD₁ and HYD₂ (facing Phe^{6,52}), one hydrogen bond acceptor group (binding to Ser^{5,42} and Tyr^{5,43}) next to HYD₁, and 5-6 Å apart from the HYD₁, and a positive ionisable moiety contacting Asp^{3,32}.

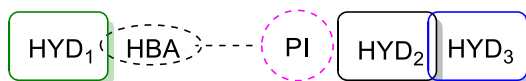


Figure 4 – General pharmacophore model of 5-HT₇R antagonists. PI: positive ionisable portion; HYD₁₋₃: hydrophobic sites; HBA: hydrogen bond acceptor feature

2.5. Serotonin receptor subtype-2B (5-HT_{2B}R)

Non-selective agonists of 5-HT_{2B}R receptor (e.g. ergolines⁹) are causing acute side-effects of hypertension, cardiac valvulopathy and retroperitoneal fibrosis. In

⁸ López-Rodríguez ML, Porras E, Morcillo MJ, et al. Optimization of the Pharmacophore Model for 5-HT₇R Antagonism. Design and Synthesis of New Naphtholactam and Naphthosultam Derivatives. *J Med Chem.* 2003;46(26):5638-5650.

⁹ Silberstein SD, McCrory DC. Ergotamine and Dihydroergotamine: History, Pharmacology, and Efficacy. *Headache J Head Face Pain.* 2003;43(2):144-166.

contrast, evidence has shown that inhibition of the receptor is beneficial in the treatment of migraine¹⁰, chronic heart disease¹¹, and irritable bowel syndrome (IBS)¹².

Most prominent 5-HT_{2B}R antagonists might be represented by a couple of chemotypes (summarized in **Figure 5**). Examples of high affinity 5-HT_{2B}R compounds showing selectivity against 5-HT_{1B}R are represented by triazines (**7**), piperidines (**8**) and aryl-ureas (SB-200646A (**9**), SB-204741, SB-215505).

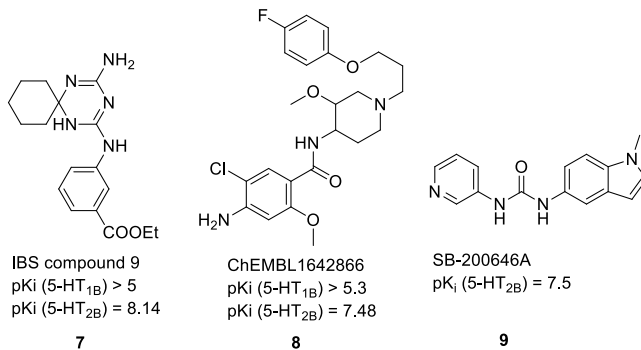


Figure 5 - Representative selective 5-HT_{2B}R ligands, Compounds **7-8** with available 5-HT_{1B}R and 5-HT_{2B}R binding affinity data, and example of clinical candidate **9**

3. EXPERIMENTAL AND COMPUTATIONAL METHODS

3.1. Computational methods

Knime.com AG's Konstanz Information Miner (Knime) data-miner and script-based workflow manager was used for the collection and mining of chemical/biochemical databases, assembly of training and validation sets for the development of ligand-, and structure-based methods, for the calculation of the desirability scores, and for the evaluation and scoring of docking-based virtual screenings. ChEMBL database (ChEMBL. <https://www.ebi.ac.uk/chembl/>), PubChem database (The PubChem Project. <https://pubchem.ncbi.nlm.nih.gov/>), and MCule database (mcule. <https://mcule.com/>) were used as sources for the collection of the studied biochemical

¹⁰ Johnson KW, Phebus LA, Cohen ML. Serotonin in migraine: theories, animal models and emerging therapies. *Prog Drug Res.* 1998;51:219-244.

¹¹ Janssen W, Schymura Y, Novoyatleva T, et al. 5-HT_{2B} receptor antagonists inhibit fibrosis and protect from RV heart failure. *Biomed Res Int.* 2015;2015:438403.

¹² Zhou Y, Ma J, Lin X, Huang X-P, Wu K, Huang N. Structure-Based Discovery of Novel and Selective 5-Hydroxytryptamine 2B Receptor Antagonists for the Treatment of Irritable Bowel Syndrome. *J Med Chem.* 2016;59(2):707-720.

datasets. Structure-based molecular dockings (altogether with preparation of ligand and protein structures) were performed by Small-Molecule Drug Discovery Suite, Schrödinger, LLC, New York, NY. Glide Single Precision dockings were performed. Physicochemical descriptors were calculated by ChemAxon's JChem for Office.

3.2. Synthetic chemistry methods

All chemical reagents and screening compounds used and studied were purchased from commercial chemical suppliers. The NMR experiments were performed at 500 MHz on a Varian VNMR SYSTEM spectrometer. The HPLC-MS measurements were performed on Shimadzu LCMS2020 LC/MS system. Flash chromatography was performed using Teledyne ISCO CombiFlash Lumen+ Rf. Purifications by preparative-HPLC were performed with Hanbon NS4205 Binary high pressure semi-preparative HPLC. High resolution mass spectrometric measurements were performed using a Q-TOF Premier mass spectrometer (Waters Corporation, Milford, MA, USA) in positive electrospray ionization mode.

3.2. Biological assays

Virtual screening hits altogether with designed and synthesized compounds were measured in radioligand binding assays against 5-HT_{1A}R, 5-HT_{2A}R, 5-HT₆R, 5-HT₇R at the Department of Medicinal Chemistry, Institute of Pharmacology, Polish Academy of Sciences, Kraków; 5-HT₆R and CB₁R measurements of the fragment library screening were performed at the Institute of Enzymology RCNS-HAS; whereas 5-HT_{1B}R and 5-HT_{2B}R measurements were performed at Grupo de Investigación "BioFarma" USC, Centro de Investigación CIMUS, Santiago de Compostela, Spain.

4. RESULTS

4.1. Physicochemical-property based scoring method for the design of aminergic fragment libraries

Computationally cheap ligand-based methodologies help to narrow the size of initial datasets of multimillion molecules in virtual screening workflows. These pre-filtered sets consisting of compounds with selected properties, or satisfying certain similarity criteria to known actives, are more likely to end up in improved hit rates during screening campaigns. Our objective was to enrich the screening database with compounds predicted to be active on aminergic GPCRs. To achieve this goal we first collected fragment-like aminergic GPCR ligands from public databases (ChEMBL). Their characteristic physicochemical property distributions were analysed to derive a scoring scheme suitable to screen compound libraries for aminergic class A GPCR ligands. A representative training set containing 2183 actives and 5000 random reference inactives) was collected from the ChEMBL database for the analysis of known aminergic fragment sized molecules. Fragments were characterized by widely used physicochemical parameters, describing the polarity and topological properties

of the molecules: such as calculated logP, logD (at pH = 7.4), polar surface area (at pH = 7.4) measures (PSA), pharmacophoric descriptors: number of hydrogen bond acceptors (nHBA), number of hydrogen bond donors (nHBD); pKa (acidic, related to the strongest center), pK_a (basic, related to the strongest center), topological descriptors: number of rotatable bonds (nRot), number of nitrogen atoms (nN), number of oxygen atoms (nOx). All of the descriptors were calculated for all of the fragments both for the active and for the reference compounds, followed by calculating the distribution properties (median, mean and standard deviation) for each variable. Differences in property-distributions were identified by visual inspection and by the analysis of medians, means and standard deviations, comparing aminergic-fragments, and ChEMBL inactive reference fragments. The comparative analysis of the distributions of several physicochemical descriptors for aminergic GPCR ligands and random reference compounds revealed that the following six descriptors are markedly different for the two sets of compounds: logD (at pH = 7.4), PSA (at pH = 7.4), pK_a (strongest basic center), number of nitrogen atoms (nN), number of oxygen atoms (nOx), and the number of rotatable bonds (nRot). A desirability function maps the value of a variable onto a continuous score in the range of [0; 1]. Desirable and undesirable property-ranges (x-axis) are defined by the function through series of inflection points¹³, identifying regions of the properties with a certain desirability score of y(x). We defined the “Fragment Aminergic GPCR Score” (FrAGS) as the sum of the individual property-scores, providing a score with a range between 0 and 6.

The selected properties were next translated to desirability functions (examples of desirability functions in **Figure 6**).

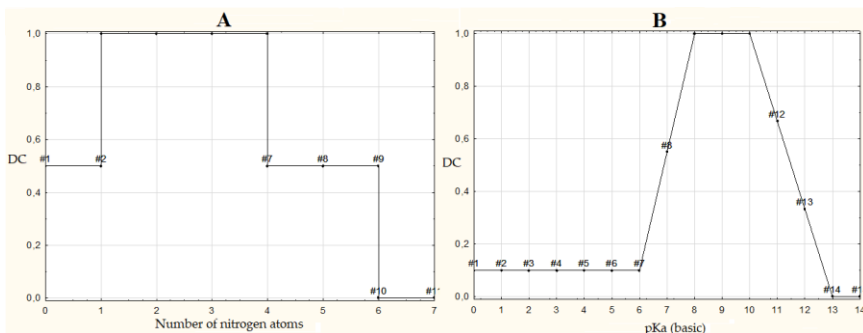


Figure 6 – Examples for desirability functions of aminergic physicochemical

¹³ Wager TT, Hou X, Verhoest PR, Villalobos A. Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties. *ACS Chem Neurosci*. 2010;1(6):435-449.

properties; Scatterplots of Desirability scores (DC) against 7/A: number of nitrogen atoms, 7/B: basic pK_a (of the strongest basic center).

The validation of the FrAGS was carried out with three complementary approaches. As a first attempt, the active training set was mixed with an order of magnitude larger random reference ChEMBL set to check whether the score is able to enrich aminergic ligands. The second approach used an independent active set from the PubChem database. The third validation used data of a high-throughput screening (HTS) and fragment screening (FS) campaigns on aminergic GPCR targets provided by Gedeon Richter Plc. The performance of the FrAGS (example presented in **Figure 7**) was measured by the calculation of Enrichment Factors (EF), Receiver Operating Characteristic (ROC) curves, and by the comparison of False Negative Rates (FNR) and True Negative Rates (TNR) as functions of the FrAGS score.

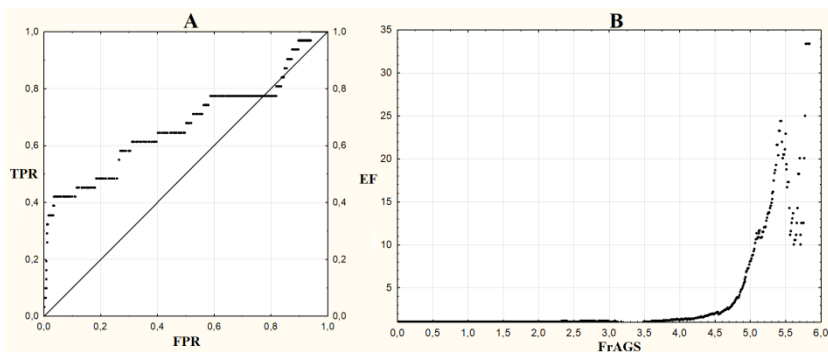


Figure 7 – ROC curve (A) and Enrichment Factor against FrAGS (B) on PubChem 5-HT_{1R} validation set.

The scoring scheme was validated with independent experimental data and it was found that a score cut-off value of 5.0 is appropriate to achieve an enrichment of around, or over 3. FrAGS is a suitable option for screening large libraries of fragment-like compounds for aminergic GPCR ligands, and thus it might be a useful tool for compiling focused fragment libraries for drug discovery projects.

4.2. Consensus-scoring based docking method for the design of aminergic fragment libraries

Since the first GPCR structure of rhodopsin was solved in 2000, an ever growing number of class-A aminergic GPCR receptor X-ray structures have been published¹⁴. The large number of available aminergic GPCR structures raises the

¹⁴ Topiol S, Sabio M. X-ray structure breakthroughs in the GPCR transmembrane region. *Biochem*

possibility for using their combinations in docking experiments to identify and assemble aminergic fragments and fragment libraries. In addition to the predicted binding affinity such a structure-based method would provide predictions on binding modes that might support further fragment optimizations. Multi-target docking is a well-known approach that is capable of achieving higher enrichments of diverse actives compared to single-target docking¹⁵. Considering the similar characteristics of aminergic orthosteric binding sites we propose, that a representative collection of aminergic GPCR structures could provide hits that may also fit to aminergic receptors not included in the screening model. However, the use of multiple structural data results in increased computational time and large volumes of data for processing. Consequently the nineteen aminergic structures available at the date of the study were clustered by the RMSD values of their binding site residues. In parallel, they were all subjected to self-docking in order to select an adequate number of representative structures suitable for docking based virtual screening. Altogether nine aminergic receptor structures were used in the optimization of the screening protocol.

The concept is based on ranking the docked entries by Glide score values per each standalone receptor structure in the ensemble. The nine different ranks belonging to a single entry were translated into votes using a ranking cut-off. The Rank(x)% criterion was defined as a percent of upper ranking boundary. The resulting votes were summed into a voting score (FrACS, Fragment Aminergic Consensus Score) with a range between 0 and 9.

Proposing the consecutive application of ligand- and structure-based methods we screened fragment-sized molecules from ChEMBL database, using the ligand-based desirability score FrAGS followed by the structure-based docking protocol FrACS in order to optimize the combined approach.

The prospective validation was carried out on our in-house fragment library by first calculating the FrAGS desirability scores, and keeping fragments with a score higher than $\text{FrAGS} \geq 5$. The desirability-scored hits of the fragment library were docked into the nine aminergic receptors. After ranking by docking scores, votes were assigned to each fragment. The 36 virtual screening hits were subjected to antagonist activity measurements in a cell-based assay against 5-HT₆R that was not included in the protein ensemble of 9 structures. We have identified four new fragment hits (**Figure 8**) with low micromolar binding affinities to 5-HT₆R and neither were described previously as 5-HT₆R receptor ligands.

Pharmacol. 2009;78(1):11-20.

¹⁵ Huang S-Y, Zou X. Ensemble docking of multiple protein structures: Considering protein structural variations in molecular docking. *Proteins Struct Funct Bioinforma.* 2006;66(2):399-421.

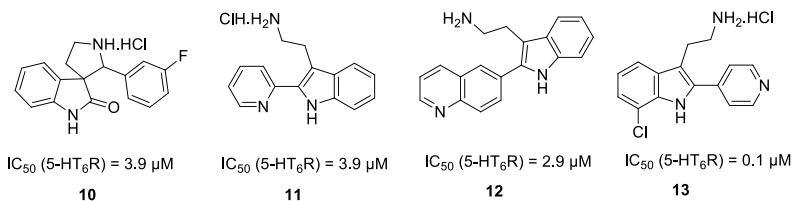


Figure 8 – 5-HT₆R hits of fragment library screening

We have accomplished a counter-screen for **compounds 11-13** against a non-aminergic target, cannabinoid receptor subtype-1 (CB₁R) in order to check specificity of compounds towards the aminergic subfamily. In fact, all of the hits were found to be inactive against CB₁R. Furthermore we have examined the four best fragment hits of the prospective validation in 5-HT₆R conformations (nine frames) obtained from the molecular dynamics (MD) simulation of its homology model¹⁶ by comparing their binding modes to a canonical 5-HT₆R antagonist SB-742457 (**1**).

The FrACS based virtual screening either itself or in combination with FrAGS is an efficient tool for the design of focused libraries for class A aminergic GPCRs and can support the identification of fragment ligands for these receptors.

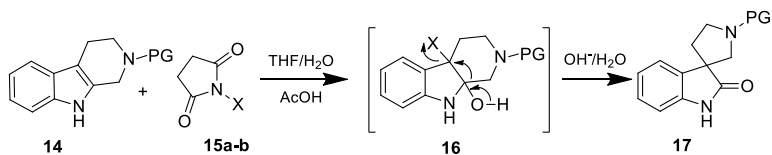
4.3. Spiro-oxindoles, and -indolines as new 5-HT₆R antagonists

The fragment hit (2'-(3-fluorophenyl)spiro[indoline-3,3'-pyrrolidin]-2-one) (**10**) was identified as a fragment hit with micromolar inhibitory activity against 5-HT₆R in the prospective validation of our combined FrAGS and FrACS approach. Optimization of the spiro[pyrrolidine-3,3'-oxindole] scaffold required a viable synthesis strategy for the preparation of designed analogues. We applied the convenient conversion of N-protected tryptolines **14** to the corresponding spiro[pyrrolidine-3,3'-oxindoles] **17** by an oxidative spiro-rearrangement reaction induced by an oxidative agent (N-chlorosuccinimide (NCS) **15a**, N-bromosuccinimide (NBS) **15b**¹⁷⁻¹⁸. The reaction is completed by the subsequent elimination of hydrogen-bromide (**16**) that finally results in the reorganization of the ring system to spiro[pyrrolidine-3,3'-oxindoles] (**17**) (**Figure 9**).

¹⁶ Vass M, Jójárt B, Bogár F, Paragi G, Keserű GM, Tarcsay Á. Dynamics and structural determinants of ligand recognition of the 5-HT₆ receptor. *J Comput Aided Mol Des.* 2015;29(12):1137-1149.

¹⁷ Finch N, Taylor WI. The Conversion of Tetrahydro- β -Carboline Alkaloids into Oxindoles. The Structures and Partial Syntheses of Mitraphylline and Rhyn Cophylline. *J Am Chem Soc.* 1962;84(7):1318-1320.

¹⁸ Shavel J, Zinnes H. Oxindole Alkaloids. I. Oxidative-Rearrangement of Indole Alkaloids to their Oxindole Analogs. *J Am Chem Soc.* 1962;84(7):1320-1321.



PG: protecting group, e.g. tert-butyloxycarbonyl (Boc), benzyl (Bn), benzyloxy carbamate (Cbz), etc.
X: **27a** Cl, **27b** Br

Figure 9 - Mechanism of the succinimide assisted oxidative spiro-rearrangement of tryptolines.

We performed a hit-to lead optimization by the pharmacophore-model¹⁹ and structure-activity relationship (SAR) analysis to explore several derivatives of the spiro[pyrrolidine-3,3'-oxindole] scaffold (**17**). Throughout the process we introduced the phenylsulfonyl moiety (**19-20**, **22-26**) and examined the corresponding reduced spiro[pyrrolidine-3,3'-indoline] scaffold (**21-26**).

The structure-activity relationship around the original (2'-(3-fluorophenyl)spiro[indoline-3,3'-pyrrolidin]-2-one) hit (**10**) was first investigated using the "SAR-by-catalog" approach. All purchasable screening compounds with the spiro[pyrrolidine-3,3'-oxindole] scaffold were collected from the Mcule database. The collected set was docked to the nine 5-HT₆R MD frames. The *in vitro* screening of the 10 best docked hits resulted in some moderate improvement in affinity and unacceptable selectivity. Therefore, we decided to explore the novel 5-HT₆R chemotype by a more conventional medicinal chemistry strategy. A synthetic tree (**Figure 10**) of the synthesized oxindoles and indolines was elaborated starting from the core tryptoline intermediate (**18**). The removal of the bulky lipophilic group at the 2'-position of the pyrrolidine ring and the insertion of the classical phenylsulfonyl moiety (**19-20**) resulted in notable selectivity across the serotonergic GPCR panel receptors (5-HT_{1A}R, 5-HT_{2A}R, 5-HT₆R, 5-HT₇R). A feature not matching the pharmacophore pattern was the hydrogen bond acceptor oxo group in the oxindole core. We therefore synthesized different 1-(phenylsulfonyl) indolines (**22**, **23a,b**, **25**), substituted at the 1'-pyrrolidine nitrogen with either lipophilic, or basic nitrogen containing groups. As a result we selected 1'-benzyl-1-(phenylsulfonyl) spiro[indoline-3,3'-pyrrolidine] (**22**) for further optimization of this chemotype. As a next step, we examined the substituent vectors at both the benzyl (**26a-c**) and the

¹⁹ Kelemen ÁA, Mordalski S, Bojarski AJ, Keserü GM. Computational Modeling of Drugs for Alzheimer's Disease: Design of Serotonin 5-HT₆ Antagonists. In: Vol 132. ; 2018:419-461.

phenylsulfonyl rings (**24a-c**) by walking fluorine substituents around²⁰. The alternative growing direction towards the phenylsulfonyl ring (compounds **24a-c**) showed improvement in the affinities, compared to the results of **22**, also retaining the good selectivity against the 5-HT_{1A}R and 5-HT₇R subtypes (200 nM affinity towards 5-HT₆R, and around 200-fold selectivity against 5-HT₇R). These data suggest position-2 of the phenylsulfonyl group of **24a** can be beneficially substituted and therefore it is worth to explore this vector further in the lead optimization phase. The improved affinity of **24a** was interpreted by docking the compound to the 5-HT₆R receptor model used previously. Docking of **24a** by Schrödinger—Glide either with, or without applying any constraint (requiring hydrogen bonding with both Asn288^{6,55} and/or Ser193^{5,43}) resulted in unchanged, and consistent docking poses inside the orthosteric binding pocket. As a result, 1'-benzyl-1-(phenylsulfonyl) spiro[indoline-3,3'-pyrrolidine] (**22**) and its corresponding 2-, 3-substituted analogues **24a-b** were identified as promising 5-HT₆R ligands.

²⁰ Shah P, Westwell AD. The role of fluorine in medicinal chemistry. *J Enzyme Inhib Med Chem*. 2007;22(5):527-540.

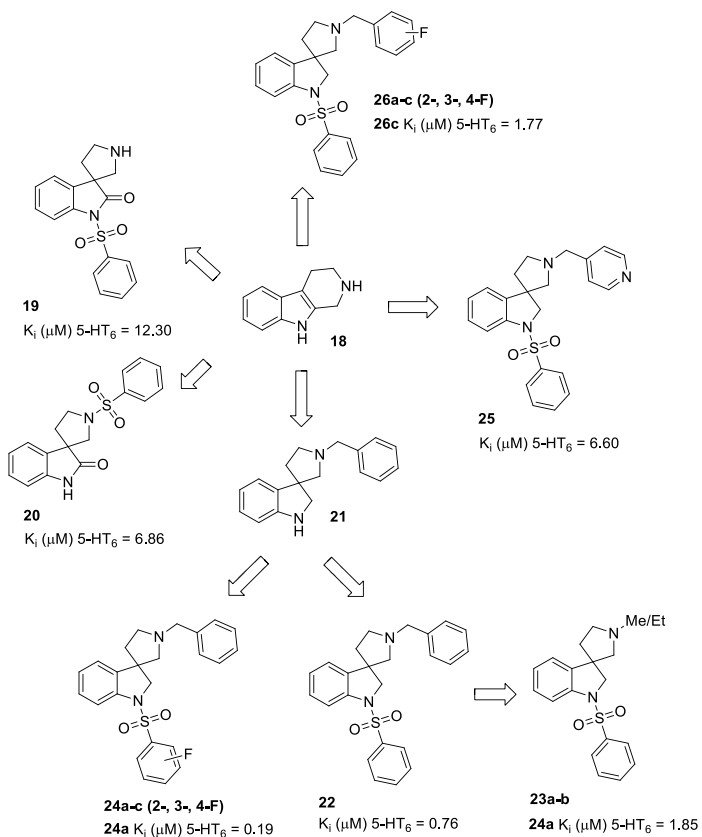


Figure 10 - Spiro[pyrrolidine-3,3'-oxindoles] and spiro[pyrrolidine-3,3'-indolines] as 5HT₆R ligands.

4.4. Spiro-oxindoles as new 5-HT₇R antagonists

As presented in **Figure 3** and **11**, the tetrahydrobenzindoles (e.g. DR-4004 **6**,²¹)

²¹ Kikuchi C, Ando T, Watanabe T, et al. 2a-[4-(Tetrahydropyridoindol-2-yl)butyl]tetrahydrobenzindole Derivatives: New Selective Antagonists of the 5-Hydroxytryptamine 7 Receptor. *J Med Chem.* 2002;45(11):2197-2206.

were validated as potent 5-HT₇R scaffolds. As an attempt to synthesize new, selective and less lipophilic compounds we designed (arylpiperazinylethyl)spiro[indoline-3,3'-pyrrolidin]-2-ones (**27**) as potential 5-HT₇R ligands (see **Figure 11**).

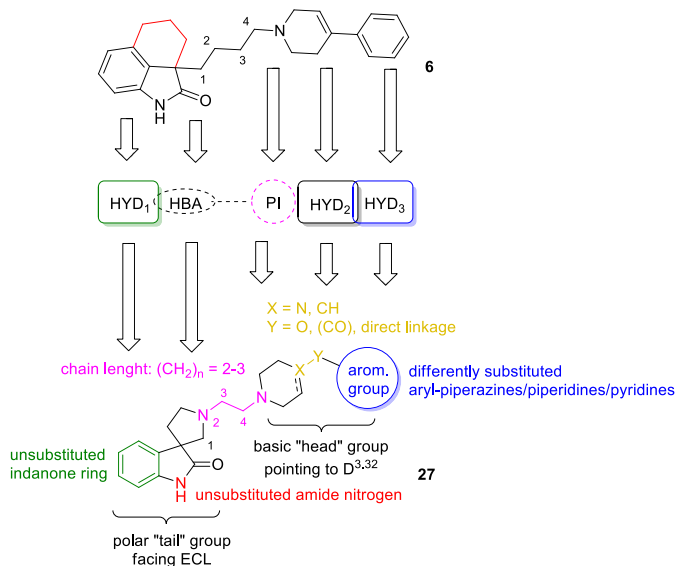


Figure 11 – Design concept for the SAR analysis of (arylpiperazinylethyl)spiro[indoline-3,3'-pyrrolidin]-2-ones (**27**).

Designing selective 5-HT₇R compounds against other serotonin receptors (5-HT_{1A}R, 5-HT_{2A}R, 5-HT₆R) we used the canonical pharmacophore models²² (**Figure 4**). These models suggest that it is beneficial to decrease the distance between PI (positive ionizable) and HBA (H-bond acceptor), to facilitate interactions to Ser^{6.55} (Ala^{6.55} at 5-HT_{1A}R), and to introduce polar substituents at HYD₁-HYD₂ to contact Arg^{7.36} (that is absent in 5-HT_{1A}R). Following these guidelines we set our objective for exploring selectivity drivers around the spiro[pyrrolidine-3,3'-oxindoles] core.

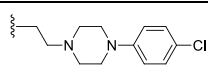
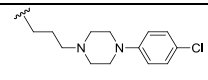
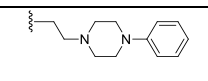
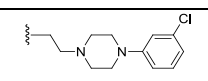
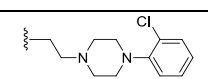
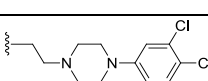
In line with other studies²³ we prepared compounds with two atom spacer (CH₂)_n(n=2), although n=3 was also synthesized (**29**) in order to investigate its' effect

²² López-Rodríguez ML, Porras E, Morcillo MJ, et al. Optimization of the Pharmacophore Model for 5-HT₇R Antagonism. Design and Synthesis of New Naphtholactam and Naphthosultam Derivatives. *J Med Chem.* 2003;46(26):5638-5650.

²³ Pittala V, Pittala D. Latest Advances Towards the Discovery of 5-HT₇ Receptor Ligands. *Mini-Reviews Med Chem.* 2011;11(13):1108-1121.

on flexibility and HBA-PI distances. Based on SAR data, the substitution of the aromatic ring either did not substantially affect or even decreased the 5-HT₇R affinity. Thus we kept our spiro[pyrrolidine-3,3'-oxindole] core unsubstituted. Halo-scan, however, was planned to explore the substituent effects around the phenyl ring of the arylpiperazine moiety comparing the profile of the unsubstituted (**30**) and the meta-Cl (**31**), ortho-Cl (**32**), para-Cl (**28**), and 3,4-dichloro-derivatives (**33**). Furthermore we plan to explore the impact of the HYD₁/HYD₂ features on the 5-HT₇R affinity and selectivity we replaced the canonical arylpiperazine by phenyl-, and phenoxy-piperidines (**34**, **35**, **38**), 5,6-dihydropyridine (**36**), and benzoyl-piperazine (**39**) moieties.

The synthesized compounds were investigated in competition binding assays against 5-HT₇R and other related serotonin receptor subtypes including 5-HT_{1A}R, 5-HT_{2A}R and 5-HT₆R (**Table 1**).

ID	Structure	5-HT _{1A}	5-HT _{2A}	5-HT ₆	5-HT ₇
		K _i (μM)	K _i (μM)	K _i (μM)	K _i (μM)
28		3.755	0.068	4.737	0.029
		129.5	2.3	163.3	
29		2.891	0.715	5.559	0.067
		43.1	10.7	83.0	
30		6.005	0.496	6.556	0.100
		60.1	5.0	65.6	
31		2.687	0.351	2.005	0.055
		48.9	6.4	36.5	
32		1.096	0.452	2.843	0.245
		4.5	1.8	11.6	
33		4.054	0.270	1.131	0.038
		106.7	7.1	29.8	

34		14.900	0.404	4,577	0.284
		<i>52.5</i>	<i>1.4</i>	<i>16.1</i>	
35		52.010	0.259	5.285	0.275
		<i>189.1</i>	<i>0.9</i>	<i>19.2</i>	
36		3.949	0.075	6.267	0.043
		<i>91.8</i>	<i>1.7</i>	<i>145.7</i>	
37		14.160	0.059	4.744	0.021
		<i>674.3</i>	<i>2.8</i>	<i>225.9</i>	
38		13.790	0.343	1.693	0.121
		<i>114.0</i>	<i>2.8</i>	<i>14.0</i>	
39		48.240	17.330	7.746	0.529
		<i>91.2</i>	<i>32.8</i>	<i>14.6</i>	

Table 1 - Serotonergic GPCR panel of the spiro-oxindole derivatives as measured in binding assays of four serotonin receptors (measured values of K_i (μM)). 5-HT₇R selectivities are shown in italics.

The unsubstituted phenyl-piperazine derivative (**30**) showed reasonably high affinity towards 5-HT₇R, however its selectivity was moderate. Halo-scan around the phenyl ring revealed that the 5-HT₇R affinity and selectivities against 5-HT_{1A} and 5-HT_{2A}R are increasing from ortho (**32**) – meta (**31**) – para (**28**) direction. In fact, the para-Cl derivate (**28**) showed low nanomolar affinity for the target and more than hundred-fold selectivity against two of the three other serotonin receptors. Selectivity against these receptors was further improved for the para-F analogue (**37**). All of these compounds have submicromolar 5-HT_{2A} affinity that might be interesting due to potentially comorbid indications²⁴. As compared to compound **28**, the longer ([CH₂]₃) alkyl chain in **29** increased the distance between the PI and HBA features, accounting for an overall loss of affinity for the 5-HT₇R. Selectivity against 5-HT_{1A}R decreased,

²⁴ Deau E, Robin E, Voinea R, et al. Rational Design, Pharmacomodulation, and Synthesis of Dual 5-Hydroxytryptamine 7 (5-HT₇)/5-Hydroxytryptamine 2A (5-HT_{2A}) Receptor Antagonists and Evaluation by [18F]-PET Imaging in a Primate Brain. *J Med Chem.* 2015;58(20):8066-8096.

however, the selectivity against the 5-HT_{2A}R subtype has improved. **30** showed lower selectivity (65.6-fold) towards 5-HT₆R than **28** (163.3-fold). Starting from the most active 4-fluorine derivative (**37**) the 5,6-dihydropyridine derivative (**36**) showed decreased selectivity (91.8-fold) against 5-HT_{1A}R. Replacing the piperazine ring by piperidine (**35**) gave 10-times lower affinity that was further confirmed by the corresponding 4-chloro analogue (**34**). Selectivities against 5-HT_{1A}R and 5-HT_{2A}R did not change significantly, but the 5-HT₆R selectivity decreased ten-times. Similar to **34** and **35**, introduction of the phenoxy-piperidine moiety (**38**) improved the affinity towards 5-HT₆R. Finally, the benzoyl-piperazine derivative (**39**) showed reduced affinity to 5-HT₇R, however, it has the highest selectivity against 5-HT_{2A}R (32.8-fold).

In summary, preliminary SAR data demonstrates that spiro[pyrrolidine-3,3'-oxindoles] can be optimized to potent 5-HT₇R ligands. We confirmed that the 2-methylene linker ensures the ideal distance between HYD₁ and HYD₂ and therefore it is beneficial for the 5-HT₇R affinity. Para substitution at the aryl-piperazine moiety is advantageous both for affinity and selectivity against 5-HT_{1A}R and 5-HT₆R. Actually, the 4-fluoro analogue (**37**) showed the best affinity and most remarkable selectivity against 5-HT_{1A}R and 5-HT₆R. Selectivity against 5-HT_{2A}R might be improved by replacing the phenyl substituent of the piperazine by a benzoyl group. These results show the potential of this novel chemotype and validate its further optimization for more detailed *in vivo* characterization in disease models.

4.5. Consensus-scoring based docking method for the design of 5-HT_{2B}R selective antagonists

A thorough analysis of the crystal structures of aminergic GPCR proteins revealed that most of the receptors have a secondary binding pocket (SBP) formed at the extracellular part of the protein by the participation of the extracellular loop 2 (ECL2). This site contains a significant proportion of non-conserved amino acids across certain aminergic GPCR's that provides an opportunity to obtain subtype selectivity. Michino and colleagues²⁵ collected all such binding cavities with at least three residues interacting with ligands outside of the canonical orthosteric binding pocket (OBP). Their analysis identified residues involved in interactions with ligands constituting the SBP in 30 out of 36 X-ray structures of aminergic receptors. These cavities consisting of non-conserved residues open the possibilities for the design of subtype-selective ligands by maintaining crucial ligand-contacts in the OBP by an initial fragment, and optimizing it by fragment-linking with subtype-specific SBP-fragments. In this study we combined Structure Connectivity Fingerprint (SCFP) based machine-learning tool with SBP-targeted docking for the design of 5-HT_{2B}R selective ligands.

²⁵ Michino M, Beuming T, Donthamsetti P, et al. What can crystal structures of aminergic receptors tell us about designing subtype-selective ligands? *Pharmacol Rev.* 2015;67(1):198-213.

First we built a NSFP fingerprint-based machine learning model¹²⁶ using *in vitro* activity data available for human 5-HT_{1B}R and 5-HT_{2B}R receptors in ChEMBL. Seven training sets were used for building machine learning models (1-2. actives, 3-4. inactives, 5-6. selectives for both 1B and 2B receptor subtypes, and 7. nonselectives). The trained model was used prospectively for the screening of the commercial database of MCule purchasable screening compounds (4.8M compounds). In the next sequential screening step, the pre-filtered 5-HT_{2B} selective compound set of 24849 compounds was subjected to two complementary docking workflows (i) considering non-conserved amino acid pairs combined with ranking-based consensus scoring, and (ii) accounting for water molecules combined with ranking-based consensus scoring. Altogether nine compounds - predicted to be 2B selective - were selected for competition binding assay for 5-HT_{1B}R, and 5-HT_{2B}R.

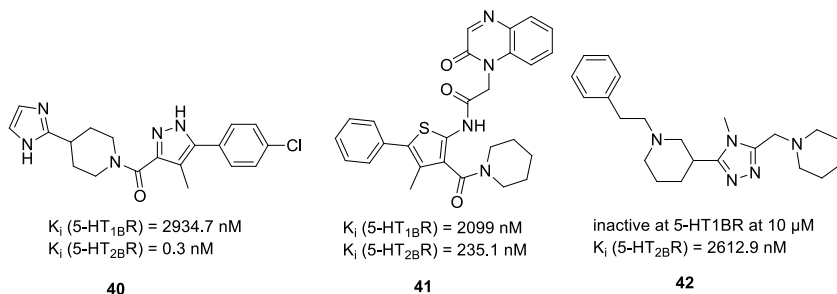


Figure 12 – New potent and selective 5-HT_{2B}R inhibitors identified by virtual screening.

Three hit compounds were identified (compounds **40-42** in **Figure 12**), showing preference towards the desired 5-HT_{2B}R target over 5-HT_{1B}R off-target. Moreover, selectivity of two hit 5-HT_{2B}R ligands **40-41** over four serotonin receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT₆ and 5-HT₇) was confirmed. The best compound (**40**) showed subnanomolar affinity and ten thousand-fold selectivity for 5-HT_{2B}R that nominates it for *in vivo* testing.

²⁶ Witek J, Smusz S, Rataj K, Mordalski S, Bojarski AJ. An application of machine learning methods to structural interaction fingerprints-A case study of kinase inhibitors. *Bioorganic Med Chem Lett.* 2014;24(2):580-585.

5. THESIS POINTS

1. A physicochemical property-based desirability scoring method (FrAGS – Fragment Aminergic GPCR Score) was developed to identify aminergic Class A GPCR ligands. This method might serve as a pre-filtering step with low computational cost for the design of fragment-libraries and for the compilation of ligand sets for structure—based virtual screenings. The performance of FrAGS was validated in retrospective and prospective applications. [K1]

2. A structure-based virtual screening method (FrACS – Fragment Aminergic Consensus Score) was developed for the design of aminergic GPCR focused fragment libraries, using an ensemble of available aminergic X-ray structures to date. The consecutive application of the FrAGS and FrACS methods was validated in a prospective screening of our in-house fragment library. As a result, four low micromolar to nanomolar 5-HT₆R hits were identified including spiro[pyrrolidine-3,3'-oxindoles] as a novel starting point for 5-HT₆R ligands. [K2]

3. The pharmacophore supported optimization of the spiro[pyrrolidine-3,3'-oxindoles] led to the identification of aryl-sulfonylated spiro[indoline-3,3'-pyrrolidines] with improved 5-HT₆R affinities. A structure-activity relationship analysis of 11 SAR-by-catalogue and 12 synthesized compounds was performed around the scaffold of spiro[indoline-3,3'-pyrrolidines] resulting in new 5-HT₆R antagonists with submicromolar affinities and 100-to-200-fold selectivities against 5-HT₇R. [K3, K4]

4. Aryl-piperazine type analogues of spiro[pyrrolidine-3,3'-oxindoles] were identified as potent 5-HT₇R ligands. A pharmacophore supported optimization led to the synthesis and identification of 12 novel 5-HT₇R ligands with double-digit nanomolar potency and highly selective (100-to-200-fold) against 5-HT_{1A}R, 5-HT_{2A}R, 5-HT₆R, 5-HT₇R.

5. Structure-based consensus scoring was applied in combination with a ligand-based machine learning pre-filter for the design of 5-HT_{2B}R selective antagonists that provided two highly potent and selective 5-HT_{2B}R ligands. [K5]

6. APPLICABILITY

The desirability function based FrAGS scoring method in combination with the docking-based consensus scoring FrACS method is an efficient tool for the design of aminergic GPCR ligands.

The presented results showed the potential of the spiro[indoline-3,3'-pyrrolidines] and spiro[pyrrolidine-3,3'-oxindoles] as novel chemotypes in the field of 5-HT₆R and 5-HT₇R inhibitors respectively, and validate their further optimization for more detailed in vivo characterization in disease models.

PUBLICATIONS

7.1. Publications related to the thesis:

K1. Kelemen Á. A.; Ferenczy Gy. G.; Keserű Gy. M. A desirability function-based scoring scheme for selecting fragment-like class A aminergic GPCR ligands. *Journal of Computer-Aided Molecular Design* **2015**, 29(1), 59-66. [IF: 3.199 (2015), I (független): 5(2)]

K2. Kelemen Á. A.; Kiss R.; Ferenczy Gy. G.; Kovács L.; Flachner B.; Lőrincz Zs.; Keserű Gy. M. Structure-Based Consensus Scoring Scheme for Selecting Class A Aminergic GPCR Fragments. *Journal of Chemical Information and Modeling* **2016**, 56(2), 412–422. [IF: 3.76 (2016), I (független): 6(5)]

K3. Kelemen Á. A.; Satała G.; Bojarski A. J.; Keserű Gy. M. Spiro[pyrrolidine-3,3'-oxindoles] and Their Indoline Analogues as New 5-HT₆ Receptor Chemotypes. *Molecules* **2017**, 22(12), 2221. [IF: 2.861 (2016), I (független): 0(0)]

K4. Kelemen Á. A.; Mordalski S.; Bojarski A. J.; Keserű Gy. M. Computational Modeling of Drugs for Alzheimer's Disease: Design of Serotonin 5-HT₆ Antagonists. *Neuromethods (Spiringer Protocols)* **2017**, 132, 419-461. ISBN: 978-1-4939-7404-7

K5. Rataj K.; Kelemen Á. A.; Brea J.; Loza M. I.; Bojarski A. J.; Keserű Gy. M. Fingerprint-Based Machine Learning Approach to Identify Potent and Selective 5-HT_{2B}R Ligands. *Molecules* **2018**, 23, 1137. [IF: 2.861 (2016), I (független): 0(0)]

7.2. Further publications:

K6. Kelemen Á. A.; Szabó B. P.; Kovács P.; Keserű Gy. M. The first synthesis of furo[2,3-c]pyridazin-4(1H)-one derivatives. *Tetrahedron Letters* **2016**, 57(1), 64-66. [IF: 2.193 (2016), I (független): 1(1)]

7.3. Presentations, posters

1. Presentation: ChemAxon User Group Meeting, Budapest, 2014. május 22.; Physicochemical property based scoring scheme for design of an aminerg GPCR targeted fragment library Fragment GPCR Score – Kelemen Ádám Andor, Ferenczy György Gábor, Keserű György Miklós
2. Presentation: Kemometriai és Molekulamodelllezési QSAR Szimpózium, Szeged, 2014, május 23.; Aminerg GPCR célzott fragmenskönyvtár-tervezésre alkalmas fizikai-kémiai leíró alapú pontozási módszer – Kelemen Ádám Andor, Ferenczy György Gábor, Keserű György Miklós
3. Poster: SCT 22nd Young Research Fellow Meeting, Franciaország, Párizs, 2015. február 4-6.; A Desirability Function-based Scoring Scheme for Selecting Fragment-like Class-A Aminergic GPCR Ligands – Kelemen Ádám Andor, Ferenczy György Gábor, Keserű György Miklós
4. Presentation: Kemometriai és Molekulamodelllezési QSAR Szimpózium, Szeged, 2015, május 14-15.; Ligandum- és szerkezet alapú módszerek aminerg GPCR célzott fragmenskönyvtárak tervezésére – Kelemen Ádám Andor, Kiss Róbert, Ferenczy György Gábor, Keserű György Miklós
5. Presentation: 10th European Workshop in Drug Design – Novel Trends in Computational Chemistry and HIV Research, Olaszország, Siena (Tuscany, Certosa di Pontignano), X EWDD, 2015. 05.16.-23.; Ligand and Structure-Based In Silico Methods for Aminergic GPCR Fragment Library Design - Kelemen Ádám Andor, Kiss Róbert, Ferenczy György Gábor, Keserű György Miklós
6. Presentation: Schrödinger Felhasználói Találkozó, Budapest, 2015.11.15.; Identification of new 5-HT6 ligands by structure-based consensus scoring – Kelemen Ádám Andor, Kiss Róbert, Ferenczy György Gábor, Kovács László, Flachner Beáta, Lőrincz Zsolt, Keserű György Miklós
7. Poster: GTC European Pharma Summit, Berlin, Germany, 2017.11.16-17. A Structure-based consensus scoring scheme for class-A aminergic GPCR fragments. Kelemen Ádám Andor, Kiss Róbert, Ferenczy György Gábor, Kovács László, Flachner Beáta, Lőrincz Zsolt, Keserű György Miklós