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Gaining in Pan-Affinity Towards Sigma 1 and Sigma 2 Receptors. SAR studies on arylalkylamines.

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Abstract

Sigma Receptor (SR) modulators are involved in different signal transduction pathways, representing important pharmacological/therapeutic tools in several pathological conditions, such as neurodegenerative diseases and cancers. To this purpose, numerous compounds have been developed in order to target selectively one of the two subtypes (S1R and S2R) as chemotherapeutic agent. However, experiments have also shown that ligands able to bind both SR subtypes can be useful for the diagnosis and/or the treatment of cancers. Therefore, the discovery of compounds with good affinity towards both S1R and S2R ("pan-modulators") is also of great interest and still represents a challenge up to now. For this reason, we synthesized novel arylalkylamines with the aim to obtain compounds with S1R and S2R affinity in the nM range and, by modelling quantitative structure-activity relationships (QSARs), we identified the essential structural features to obtain promising pan-compounds.

Keywords: sigma receptors, arylalkylamine, pan-affinity, QSAR.

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1. Introduction

The term Sigma Receptor (SR) was coined in 1976 to identify a new opioid receptor subtype; Martin et al. showed the high affinity presented by the benzomorphan analog (±)-SKF-10,047 (**Figure 1**) toward this receptor subtype class.[1] Subsequent studies established that the previous classification was not proper, since the opioid antagonists, naloxone and naltrexone, were ineffective toward SR.[2-4] Another hypothesis, mistakenly described, proposed SR as the binding site of phencyclidine (**Figure 1**), located on the ionic channel associated to the N-methyl-D-aspartate (NMDA) receptor.[5]

Two subtypes have been discovered so far, Sigma 1 Receptor (S1R) and Sigma 2 Receptor (S2R), with different distribution and pharmacological/pathological behavior.[6, 7]

The gene encoding S1R, cloned in 1996, expresses an integral membrane protein composed by 223 amino acids, resulting in a molecular weight of 23-30 kDa. S1R is highly conserved among different animal species, that share a sequence similarity of 90-96 %.[8-10] These data, collected during the years, laid the foundation for accessing to the first three-dimensional (3D) model of S1R through homology modeling techniques and to the design of numerous ligands[11]. Indeed, the structural model allowed the design of several compounds with a good binding profile toward S1R and the rationalization of the binding results that were obtained by ligand-based drug design.[12, 13]

Only in 2016, the crystal structure of the human S1R has been determined in complex with two ligands endowed with high S1R affinity (pdb codes 5hk1, 5hk2): it is constituted by a trimer, with a single transmembrane helix and a cytosolic domain for each monomer. The ligand binding pocket is placed in the β-barrel region of the cytosolic domain and is constituted mainly by hydrophobic residues.[14] The binding is triggered by an ionic interaction with a highly conserved Glu residue (E172), that is involved in a network of hydrogen bonds with Asp126 and Tyr103. Therefore, only positively charged molecules show S1R activity. Moreover, ligands form hydrophobic π-π interactions with Tyr103 and other hydrophobic amino acids in the binding site. The previous homology model presents high degree of similarity with the crystal structure. However, the presence of a single transmembrane domain is a structural motif in disagreement with the constructs reported by antecedent studies [11, 15]. These results represent an important starting point for deepening the knowledge about this poorly understood molecular target.

From a biological point of view some questions are still open; only in the last decade several studies focused their attention on the transduction signal cascades associated with S1R. It is localized in a region, called MAM (Mitochondria-Associated-ER Membrane) domain, between the ER and the mitochondria interface.[16] In physiological conditions, S1R is associated with BiP (Binding immunoglobulin Protein) as a silent complex; instead, under stressful conditions or pharmacological manipulation, the receptor acts as molecular chaperone, controlling a broad network of proteins (voltage and ligand-gated channels, G-protein coupled receptors, kinases) and interrupting the cellular death.[17, 18] Its protective action can be explained by decreasing the concentration of reactive oxygen species (ROS) produced by mitochondria, through still unclear mechanisms. Another important role played by S1R is related to its capability to promote the communication among different cellular districts, regulating the membrane lipid composition.[19, 20]

In contrast, little information on S2R is available; the gene encoding this receptor has not been cloned yet and the protein has not been purified. It has been identified by photoaffinity labeling, using tritiated 1,3-di(2-tolyl)-guanidine ([^{3}H]DTG) (**Figure 1**): results revealed a protein of 18-21 kDa.[7] Considering these evidences and the unsuccessful attempt to identify the endogenous ligand, the design of selective S2R molecules represent still a challenge today. Recently, the Progesterone Receptor Membrane Component 1 (PGRMC1) has been proposed as S2R binding site and partially crystallized (pdb code 4x8y).[21, 22] Despite some experimental data support this theory, Chu and coworkers

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recently stated that the genes encoding S2R and PGRMC1 are different. Indeed, sequence alignment of PGRMC1 and S1R reveals sequence similarity below 30%, indicating a low probability to be homologs. Also a structural comparison shows different folds and putative binding sites. Even though PGRMC1 is supposed to be anchored to the membrane by an α helix as S1R, its crystallized cytosolic domain is not trimeric, but only upon binding to haem is able to form a dimer. Therefore, the S2R putative binding site would be highly different with respect to S1R. In contrast, S2R binding assays show that S1R and S2R ligands have similar chemical properties, indicating that the binding sites should be also similar. In conclusion, further investigations are still necessary to provide a better understanding of the S2R binding pocket.[23]

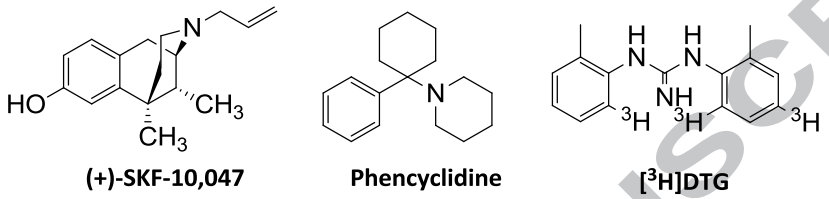

Figure 1: chemical structures of the benzomorphan analog (±)-SKF-10,047, NMDA antagonist phencyclidine and tritiated 1,3-di(2-tolyl)-guanidine.

From a pharmacological standpoint, S1R is closely related to the Central Nervous System and involved in neuroprotection [24-26]: accordingly, two S1R ligands are currently in phase II of clinical trials as potential drugs for the treatment of Alzheimer's disease and neuropathic pain respectively, the S1R agonist ANAVEX 2-73 and the S1R antagonist S1RA (**Figure 2**)[27, 28]. Furthermore, recent studies evidenced the potential in cancer therapy of S1R antagonist. Indeed, S1R is overexpressed in lung, breast and prostate cancer cell lines.[29] Also S2R is linked to several cancerous conditions [30-32], making selectiveS2R ligands useful tools in tumor diagnosis and S2R selective agonists useful in cancer treatment.

To date, the molecular panorama related to S2R modulators is wide. They belong to four main chemical classes: i) 6,7-dimethoxytetrahydroisoquinoline derivatives; ii) granatane- or tropane-related bicycle structures; iii) indole derivatives; iv) cyclohexylpiperazine analogs. Although they represent promising pharmaceutical/therapeutic tools, only compound [^{18}F]ISO-1, a PET marker of cell proliferation, is in phase I clinical trial (**Figure 2)**.[33, 34]

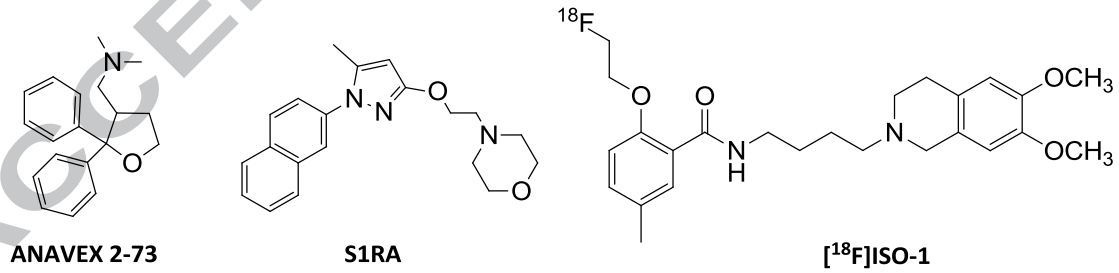

Figure 2: chemical structures of S1R and S2R ligands that are currently in clinical trials.

During the years we focused our attention on SR modulators, preparing and characterizing a wide compound library of SR ligands. These molecules possess a common arylalkyl(alkenyl)amine scaffold. Among them, **RC-33** (1-[3-(1,1'-biphen)-4-yl]-butylpiperidine) showed excellent S1R affinity (K_i S1R = 0,70 ± 0,3 nM), selectivity over S2R (K_i S2R/ K_i S1R = 147,1) and good *in vitro* metabolic stability (**Table 1**). [35-39] Considering the discovery of a new S1R *lead*

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compound (**RC-33**) and keeping in mind the high interest in S2R modulators as promising therapeutic tools, in this paper we present our efforts in better understanding the structure activity relationships (SARs) of novel **RC-33** analogs. In detail, we investigated the relevance of functional groups in obtaining a gain of affinity towards both receptors. Our aim is the discover of molecules with mixed affinity. Taking into account our reported molecules and especially compound **RC-33** [35], we designed and synthesized a small compound library (Scheme 1), in order to examine the importance of the aryl group and the amine moiety. Moreover, to better identify the chemical properties that are essential for improving the binding affinity, we generated a quantitative structure-activity relationship (QSAR) model, based on the activity data published in literature and our in-house library. This model has been, afterwards, tested on the new class of compounds that we present here.

2. Results

2.1 Chemistry

The synthesis of arylalylamines **10-13 a-d** followed the synthetic pathway reported in Scheme 1. The key step is the C-C bond generation, consisting in the nucleophilic addition of the corresponding aryl-lithium reagents **1-4** to carbonyl group of the appropriate β-aminoketone **5a-d** (**Scheme 1**). The intermediates **5a-d** were prepared *via* Michael addition of the corresponding secondary amine to but-3-en-2-one, according to the methodology reported in our previous works (anhydrous toluene at reflux or PEG 400, r.t.)[37] Once prepared, **5a-d** were added to the appropriate aryllithium reagents (generated by aryl bromine **1-4** through Br/Li exchange using *t*-butyllithium at -78 °C) to give the corresponding tertiary aminoalcohols. Without any purification, the alcohols were *in situ* dehydrated under acidic condition (37% HCl, stirring at r.t. for 12 h), thus providing the desired compounds **6-9 a-d**. The elimination reaction of alcoholic intermediates resulted highly regio- and (*E*)-stereoselective for all alkenylamines synthetized, as confirmed by ^{1}H-NMR analysis and NOESY experiments of crude compounds, in accordance with our previous experience. Arylalkenylamines **6-9 a-d** obtained as (*E*/*Z*)-mixture after chromatographic purification or crystallization could be converted into (*E*)-alkenylamines in satisfactory yields (30-77%). The final step of our synthetic strategy consisted in the conversion of **6-9 a-d** into the corresponding arylalkylamines **10-13 a-d** by catalytic hydrogenation of C-C double bond under hydrogen atmosphere using Pd(0) EnCat^{TM} 30NP. In this way, arylalkylamines **10-13 a-d,** easily isolated by solid phase extraction (SPE, SCX cartridge), were obtained with acceptable yields (43% - 95%) and in suitable amounts for the biological investigations.

6-13 a-d structures were confirmed by ^{1}H-NMR and MS analysis.

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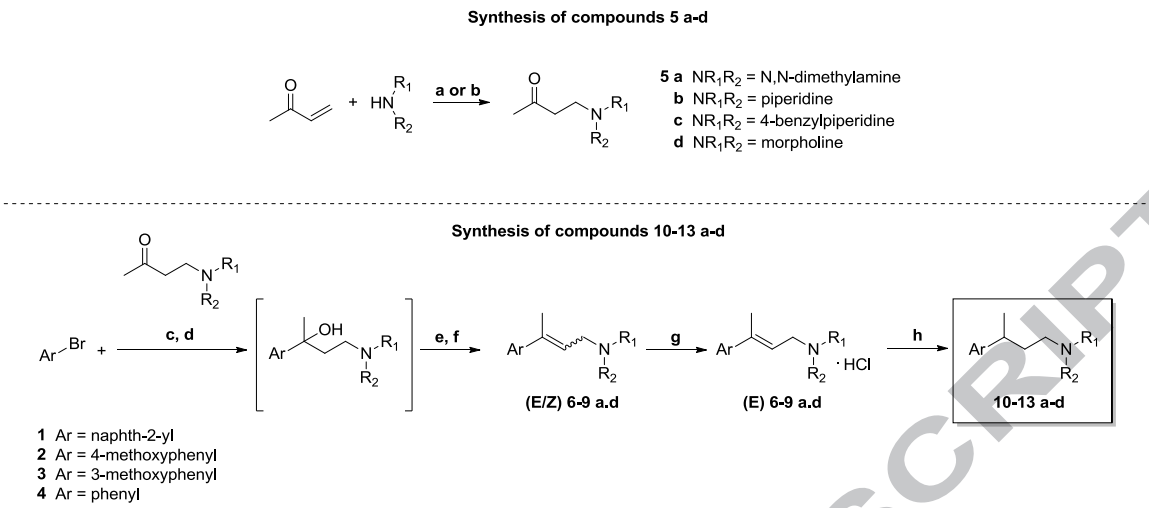

Scheme 1: Synthesis of β-aminoketones **5 a-d**. Reagents and conditions: a) anhydrous toluene, reflux for **5 a**; b) PEG 400 for compounds **5 b-d**. Synthesis of compounds **10-13 a-d**. Reagents and conditions: c) *t*-BuLi, anhydrous Et_2O, -78 °C to rt; d) ketone **5 a-d**, -78 °C to r.t.; e) 37% HCl, r.t.; f) 1N NaOH; g) crystallization from acetone; h) H_2, Pd(0) EnCat™ 30NP, abs EtOH, rt.

2.2 Binding assays

We measured the affinity towards S1R and S2R of our new compounds **10-13 a-d** through radioligand receptor binding studies. The assay for S1R is based on the use of membrane from guinea pig cerebral cortex, which represents a receptor source in the presence of a potent and selective S1R radioligand (i.e. [^{3}H]-(+)-pentazocine). Nonspecific binding values were determined using non-radiolabeled (+)-pentazocine and haloperidol in large excess. Instead, in the case of S2R, we used the membrane of rat liver as receptor source. This test was performed using a nonselective radioligand ([^{3}H]-DTG), since no S2R selective radioligand are commercially available. Moreover, it is important to mask the S1R: for this reason, an excess of non-tritiated (+)pentazocine was added to the assay solution. In order to determine nonspecific binding, a high concentration of non-tritiated DTG was used[7, 40].

Table 1 reports S1R and S2R affinities of all tested compounds in their racemic form, in comparison with the affinity of **RC-33** as reference compound. With the only exception of compound **13a**, which presents weak affinities toward both receptor subtypes, all compounds generally show from modest to good S1R affinity. Naphthalene and 4-benzylpiperidine derivatives (**10a-d**) exhibit the best S1R affinities for the presence of bulky aromatic portion, which fits well in the receptor pocket (**Figure 3**). Moreover, 4-benzylpiperidine derivatives (**11c, 12c, 13c**) show also interesting S2R affinity values. In this case, a bulky amine moiety constitutes the main feature for interacting with the S2R binding site. Indeed, N,N-dimethylamine derivatives (**11a, 12a, 13a**), presenting a small amine moiety, are characterized by a very weak affinity toward S2R. Lastly, we identified also compounds (**11d, 12b, 12d, 13b**) with mixed affinity toward both receptors subtypes, from now on called pan-selective SR ligands.

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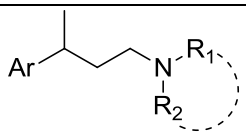
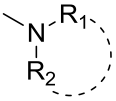
Table 1. Binding affinities towards S1R and S2R. Values are expressed as mean ± SEM of three experiments.



Compound	Ar		*Ki* S1R (nM) ± SEM	*Ki* S2R (nM) ± SEM	S2R / S1R
(*R*/*S*)-RC-33	4-biphenyl	Piperidine	0,70 ± 0,3	103 ±10	147,1
(*R*/*S*)-10a	napht-2-yl	N,N-dimethylamine	1.95 ± 0.2	43.8 ± 5.2	22
(*R*/*S*)-10b	napht-2-yl	Piperidine	1.5 ± 0.6	50 ± 6.4	33.3
(*R*/*S*)-10c	napht-2-yl	4-benzylpiperidine	19 ± 2.1	144[a]	7.6
(*R*/*S*)-10d	napht-2-yl	Morpholine	5.4 ± 1.4	33 ± 2	6.1
(*R*/*S*)-11a	4-methoxyphenyl	N,N-dimethylamine	116 ± 22	255[a]	2.2
(*R*/*S*)-11b	4-methoxyphenyl	Piperidine	20 ± 5.8	58 ± 9.4	2.9
(*R*/*S*)-11c	4-methoxyphenyl	4-benzylpiperidine	3.5 ± 0.4	18 ± 4.4	5.14
(*R*/*S*)-11d	4-methoxyphenyl	Morpholine	76 ± 7.0	68 ± 13	0.89
(*R*/*S*)-12a	3-methoxyphenyl	N,N-dimethylamine	239[a]	864[a]	3.62
(*R*/*S*)-12b	3-methoxyphenyl	Piperidine	36 ± 4.1	35 ± 4.8	0.97
(*R*/*S*)-12c	3-methoxyphenyl	4-benzylpiperidine	2.9 ± 0.7	14 ± 1.4	4.83
(*R*/*S*)-12d	3-methoxyphenyl	Morpholine	137 ± 40	92 ± 0.2	0.67
(*R*/*S*)-13a	phenyl	N,N-dimethylamine	427[a]	> 1000[a]	N.D.
(*R*/*S*)-13b	phenyl	Piperidine	46 ± 6.2	56 ± 9.2	1.22
(*R*/*S*)-13c	phenyl	4-benzylpiperidine	2.1 ± 1.0	6.5 ± 3	3.1
(*R*/*S*)-13d	phenyl	Morpholine	85 ± 6.3	71 ± 3.2	1.2

[a] Compounds with high affinity were tested three times. For compounds with low SR affinity (> 100 nM), only one measure was performed.

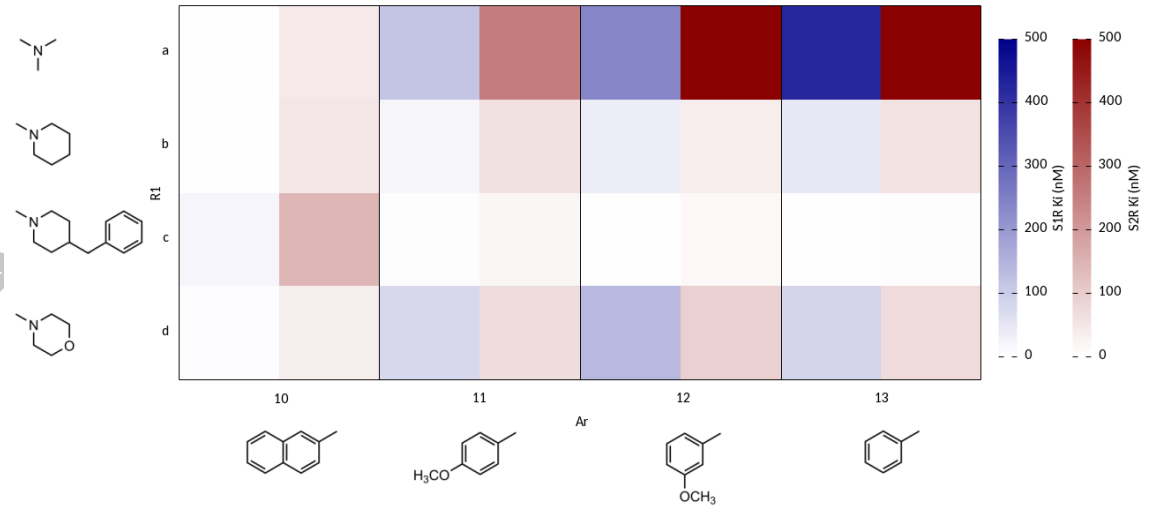


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Figure 3: comparison of S1R and S2R Ki binding affinity values. High affinity towards S1R (indicated by white to light blue colors) is achieved by naphthalene and 4-benzylpiperidine derivatives. Whereas, the highest affinity towards S2R (marked with white to light red colors) is achieved by 4-benzylpiperidine derivatives only.

2.3 QSAR Modelling

Based on previously published affinity data, we modelled quantitative structure-activity relationships (QSARs) in order to rationally interpret the experimental data and to design further ligands.

From the linear regression models we found that the binding affinity to the receptors is increased mainly by two molecular features: flexibility (expressed by "b_rotN") and hydrophobicity (expressed by "BCUT_SLOGP_3"). On one hand, flexible molecules are characterized by higher number of rotational bonds: therefore, they can easily orient in the binding pocket in order to form molecular interactions with key residues. On the other hand, the descriptor that calculates hydrophobicity (SlogP) takes additionally into consideration if determined atoms are bonded and their atomic distance. For instance, if the nitrogen atom is placed between two aromatic rings (4-benzylpiperidine series), the BCUT_SLOGP_3 value is high (~2.738) and results in better binding affinity towards both receptors; whereas, if one aromatic and one aliphatic ring (piperidine) are present, its value is lower and the binding affinity is modest. Dimethylamines show the lowest values of hydrophobicity and binding affinity. Compounds **10a** and **10b** constitute exceptions, as they possess high affinity towards S1R despite their relatively low values of BCUT_SLOGP_3. Indeed, this descriptor is identified as more important for binding to S2R than S1R, and the correlation between pK_i and BCUT_SLOGP_3 is higher for S2R than S1R (R 0.61 for S2R, 0.59 for S1R).

Moreover, given hydrophobic molecules, we assume that their solvation energy (E_sol) is higher than for water soluble compounds and, consequently, increases pK_i values for both receptors. Indeed, morpholine derivatives show the lowest energy values, whereas 4-benzylpiperidine derivatives the highest ones.

Another molecular descriptor identified by our QSAR model is globularity (expressed by "glob"), that indicates if compounds have a spherical, flat or rod-like shape: in this case, our models reveals that the binding affinity is disfavored by higher globularity values, that reveal spherical shapes. Therefore, it is clear that planar or rod-like compounds fit better to the binding site.

The binding affinity is also decreased by high dipole moment (expressed by "dipole"), that is calculated from the partial charges of the molecule. For instance, compounds of series a (dimethylamines) are characterized by high dipole moment, whereas compounds of series c (4-benzylpiperidines) by low dipole moment. This descriptor is more relevant for binding to S2R than S1R receptor, that correlates better to the ionization potential (expressed by "AM1_IP"): for instance, the compounds with a notable S1R affinity (**10a** and **10b**), have lower values than the other ligands (**13a**). Furthermore, the binding affinity is penalized by H-bond donors that do not include basic atoms like nitrogen: indeed, "a_don" counts only for atoms that are both H-bond donor and acceptors such as the hydroxyl group. The compounds that we present in this paper do not contain any OH group; however, we built the QSAR models on a library that included also compounds with an OH group attached to the alkylic chain. Comparing their affinity values, we can conclude that in average the presence of this H-bond donor feature does not improve, but instead decreases the affinity to both receptors.

We did not include the descriptor for the number of nitrogen atoms because it is a common feature to all compounds in the series and it is well known that a positively charged atom is essential for binding both receptors. Therefore, it would not add any information and improve the quality of the models.

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3. Discussion

The identification of pan-modulators, i. e. compounds that are able to bind both SR subtypes, is of great interest for the development of chemotherapeutic drugs targeting SRs. We report here the design and synthesis of pan-modulators in the class of arylalkylamines. We designed a series of **RC-33** analogues, in order to deepen the role played by the hydrophobic ring and the basic portion, maintaining the spacer bridging the two main structural features, in the interaction with the molecular targets.

The synthetic protocol provides few steps for accessing to the final alkyl-compounds. The lithium chemistry was essential to obtain the alcoholic precursors. Indeed, as reported in our previous publication[37], a Li/Br exchange, at the aromatic ring, guaranteed the lithiated species formation. The subsequent quenching with the appropriate β-aminoketone, led to crude alcohols. Without any additional manipulation, 37% HCl was added at the reaction environment. Therefore, the dehydration reaction gave the (*E/Z*) stereoisomer mixture, which was subjected to a purification, using crystallization or chromatographic purification methods, in order to obtain the (*E*)-compounds, as only isomer. A hydrogenation allowed accessing to the desired compounds **10-13 a-d**, which were obtained in good/modest yields and in sufficient amounts to perform the biological investigations.

Their affinity to S1R and S2R of **10-13 a-d** compounds were evaluated has been measured through binding assays and compared with the RC-33 respective values, in order to understand which structural changes improve the affinity toward S1R or S2R and which ones are necessary to decrease the S2R / S1R ratio.

First of all, mixed affinity is obtained by the presence of the nitrogen atom, that is expected to be charged and to form ionic interactions with Glu172 in the binding site of S1R. As the sequence of S2R is not known yet, we suppose that also the binding pocket of S2R includes an acidic amino acid that can interact with the nitrogen.

Another essential molecular property is hydrophobicity: the nitrogen atom is placed between two hydrophobic features, that can be either aromatic or aliphatic. Aromatic rings are expected to form π-π interactions with aromatic residues in the binding site, as Tyr103 in S1R. Also the binding to S2R requires two hydrophobic features around the nitrogen atom: if one of these is missing (as in the dimethylamine derivatives) the binding affinity is penalized. Although S1R receptor is also likely to bind two hydrophobic features, it does not require a second feature if the first is a naphthalene (compounds **10a-d**). Indeed, the S2R/S1R Ki ratio clearly shows that compounds **10a-d** are selective towards S1R (**Figure 4**). Therefore, to gain in affinity to both SRs, the naphthalene moiety has to be excluded.

Series **c**, i.e. 4-benzylpiperidine derivatives, is relatively more selective towards S1R than S2R, but the binding affinities are very good in both cases, indicating that the presence of a second aromatic ring bound to the piperidine favors mixed binding properties.

On the other hand, **11-12-13d** are the least selective ligands, as they present a morpholine and an aryl. Indeed, these compounds can bind both receptors for the presence of the nitrogen and the aromatic ring, but they show low binding affinity because of lower hydrophobicity and solvation energy.

Methoxy substituent does not change substantially the selectivity: for piperidines the S2/S1 ki ratio decreases only if the substitution is in meta (**12b**), for morpholine in both cases (**11d** and **12d**), whereas for 4-benzylpiperidines it increases resulting in higher selectivity towards S1R (**11c** and **12c**).

It has to be pointed out that N-(3-(3-fluorophenyl)propyl)pyrrolidine, compound number 44 from Banister et al.[41]), shows similar binding affinity towards S2R, but inactivity towards S1R (K_i S2R = 39 nM, S1R K_i >10 μM, S1R Ki/ S2R Ki = 256). Its molecular structure is similar to **12b** with a F atom in meta position of the phenyl ring and pirrolidine instead of piperidine. This confirms the importance of the substituent in meta position rather than in para to improve S2R binding affinity. On the other side, the pyrrolidine ring is more rigid than a piperidine and the fluorine

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atom acts as H-bond acceptor beside its hydrophobic properties. Therefore, these features should be investigated also on piperidine derivatives to explain the complete loss of affinity towards the S1R receptor.

In summary, our data clearly show that a satisfactory compromise between affinity and selectivity is achieved by the 4-benzylpiperidine derivatives (series c). Moreover, molecules with a piperidine or a morpholine (series b and d respectively), with the exception of the naphthalene derivatives (**10b** and **10d**), lose in affinity toward both receptor subtypes, however they maintain a good selectivity (< 3). Whereas molecules with a small amine group as N,N-dimethylamine (series a) exhibit unsatisfactory binding values towards both receptors, unless compound **10a** possess a high affinity toward S1R. From this analysis we conclude that the driving force to obtain a SR pan-modulator is represented by the right choice of the aminic moiety.

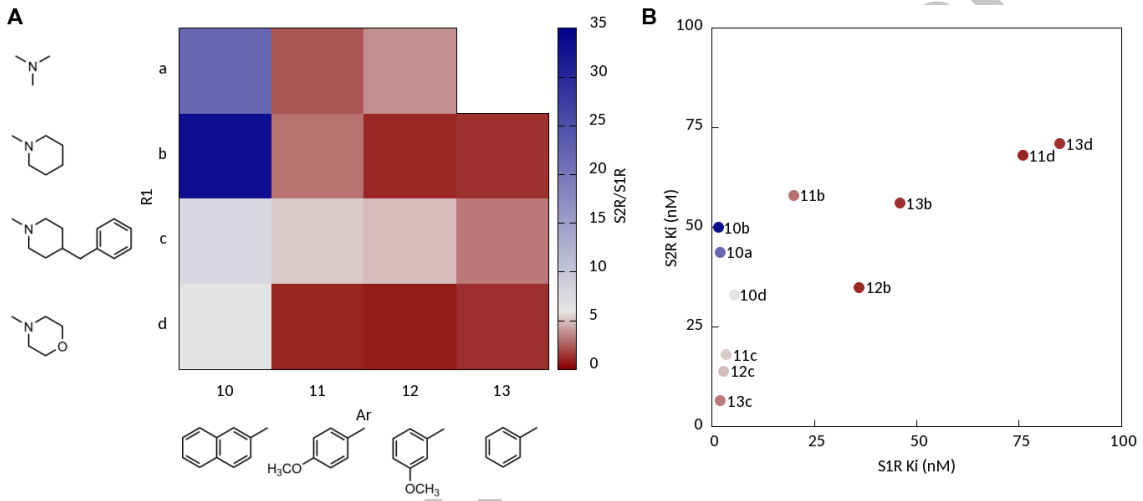

Figure 4: S2R/S1R Ki ratio (**A**) in relation to S1R and S2R affinity values (**B**). **A:** compound 13a is not shown as its S2R Ki value has not been determined with accuracy. Substitutions at amine and aromatic positions are shown and colored according to the S2R/S1R Ki ratio. Low values (marked by dark red color) indicate mixed affinity of compounds towards both receptors. Instead, selective compounds show high ratio values (highlighted by dark blue color). It is clear that enhanced mixed affinity cannot be achieved by naphthalene derivatives, that are the most active towards S1R.

B: compounds with both S1R and S2R affinity values lower than 100 nM are displayed and colored according to the S2R/S1R Ki ratio. It is evident that all 4-benzylpiperidine derivatives (series c), but the naphthalene substituted (10c), present the higher affinities towards both receptors, as they cluster in the bottom left corner of the plot. However, their S2R/S1R ratio is in the range 3 – 8, as indicated by their red to grey color; whereas morpholine and piperidine compounds (series b and d), excluding the naphthalene substitution, show very good mixed properties (marked by dark red color), but also low affinity values towards both receptors, as they cluster in the top right corner of the graph.

4. Conclusions

A novel series of arylalkylamines has been prepared and their affinity towards S1R and S2R evaluated. To clarify the structural features leading to the affinity for both receptor subtypes, robust QSAR models have been developed. QSAR modelling revealed that the substitution of the nitrogen with a benzylpiperidine allows the achievement of pan-activity

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without losing in affinity. The only exception is compound **10c**: despite the high hydrophobicity, the presence of a bulky aromatic portion, as a naphth-2-yl, causes a loss in affinity toward S2R.

Noteworthy is compound **13c**, which represents a good compromise between affinity (*K*i S1R = 2.1 ± 1.0; *K*i S2R = 6.5 ± 3) and pan-activity (S2R / S1R = 3.1). Therefore, **13c** could be considered the *hit* compound of this pan-ligand series. To sum up, our study opens the way to the design of further pan-modulators as potential novel chemotherapeutic candidates. It is important to underline that all compounds possess a stereogenic center and at this stage of the research have been tested as racemate. Our current efforts are directed to obtain homochiral compounds, to investigate the role of chirality in the interaction with the SRs.

5. Material and Methods

5.1 Chemistry

General remarks: Reagents and solvents for synthesis were obtained from Aldrich (Italy). Solvents were purified according to the guidelines in Purification of Laboratory Chemicals.[42] Melting points were measured on SMP3 Stuart Scientific apparatus and are uncorrected. Analytical thin-layer-chromatography (TLC) was carried out on silica gel precoated glass-backed plates (Fluka Kieselgel 60 F254, Merck) and on aluminiumoxid precoated aluminium-backed plates (DC-Alufolien Aluminiumoxid 60 F254 neutral, Merck); visualized by ultra-violet (UV) radiation, acidic ammonium molybdate (IV), or potassium permanganate. Flash chromatography (FC) was performed with Silica Gel 60 (particle size 230–400 mesh) purchased from NovaChimica and neutral aluminium oxide (particle size 0.05-0.15 mm) purchased from Fluka. Bond Elute SCX cartridges were purchased from Varian. IR spectra were recorded on a Jasco FT/IR-4100 spectrophotometer; only noteworthy absorptions are given. ^{1}H-NMR spectra were measured with an AVANCE 400 spectrometer Bruker, Germany at rt. Chemical shifts (d) are given in ppm, coupling constants (J) are in Hertz (Hz) and signals are designated as follows: (s) singlet, (br s) broad singlet, (d) doublet, (t) triplet, (q) quartet, and (m) multiplet. TMS was used as internal standard. MS spectra were recorded on a Finnigan LCQ Fleet system (Thermo Finnigan, San Jose, CA, USA), using an ESI source operating in positive ion mode. The purities of target compounds were determined on a Jasco HPLC system equipped with a Jasco autosampler (model AS-2055 plus), a quaternary gradient pump (model PU-2089 plus), and a multiwavelength detector (model MD-2010 plus). For the HPLC analysis of the arylalkylamines a Chromolith® column (50 x 4.6mm), eluting with H_2O (0.1% formic acid, solvent A) and ACN (0.1% formic acid, solvent B) under gradient condition (Methods A, B, C, D) at room temperature. Method A (compounds **10b-10d**, **11b**, **12c**, **13b-13d**): 0 min 95% A and 5% B, 3 min 95% A and 5% B, 13 min 5% A and 95% B, 15 min , 5% A and 95% B, 20 min, 95% A and 5% B. Flow rate 1.5 mL/min. Method B (compounds **11c-11d**, **12d**, and **13a**): 0 min 95% A and 5% B, 3 min 95% A and 5% B, 23 min 5% A and 95% B, 25 min , 5% A and 95% B, 30 min, 95% A and 5% B. Flow rate 1.5 mL/min. Method C (compound **10a** and **12b**): gradient conditions as Method B, Flow rate 2 mL/min. Method D (compounds **11a** and **12a**): 0 min 90% A and 10% B, 10 min 10% A and 90% B, 20 min 10% A and 90% B, 25 min, 90% A and 10% B. Flow rate 2 mL/min. For the general procedure and characterization of compounds **6-9 a-d** see Supplementary material.

5.1.1 General procedure for the preparation of compound **10-13 a-d**

Before use, Pd(0) EnCat™ 30NP (supplied as a water wet solid with water content 45% w/w) was washed thoroughly with absolute ethanol to remove water. Pre-washed Pd(0) EnCat™ 30NP (0.20 equiv) was added to a stirred solution of the appropriate arylalkenylamine as free base (0.14 mmol) in absolute ethanol (11 mL) and the reaction mixture was left at room temperature in hydrogen atmosphere (balloon) for 30 h. The catalyst was then filtered off and washed with

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absolute ethanol; the organic phases were lastly dried in vacuo. In this way, pure **13b** was obtained as yellow oils. In the case of compounds **10-12 a-d**, **13a** and **13c** the crudes were loaded on SCX cartridge and eluted with 1 M NH3 in methanol, pure compounds were obtained in good yield.

(R/S)-N,N-dimethyl-(3-naphthalen-2-yl-butyl)-amine ***[(R/S)-10a]****:* Yield: 59%, white solid, mp: 177-179 °C; IR (cm^{-1}): 3362, 3010, 2776, 2577, 2467, 1599, 1474, 1190, 1014, 963, 751; ^{1}H-NMR (400 MHz) (CD_3OD) δ (ppm): 7.80 (m, 3H), 7.63 (s, 1H), 7.40 (m, 3H), 2.87 (m, 1H), 2.30 (m, 2H), 2.18 (s, 6H), 1.89 (m, 2H), 1.35 (d, *J* = 7.1 Hz, 3H)); ESI-MS m/z = 228.21 $[M + H]^+$. HPLC tR = 5.42 min, > 98 % purity (λ = 270 nm).

(R/S)-1-(3-naphthalen-2-yl-butyl)piperidine ***[(R/S)-10b]****:* Yield: 77%, white solid; IR (cm^{-1}): 3050, 2928, 1908, 1600, 1122, 816, 742; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.83-2.76 (m, 3H), 7.60 (s, 1H), 7.48-7.26 (m, 3H), 2.90 (sextuplet, *J* = 7.1 Hz, 1H), 2.44-2.26 (m, 4H), 2.25-2.17 (m, 1H), 1.97-1.83 (m, 3H), 1.62-1.57 (m, 4H), 1.37-1.25 (m, 2H), 1.24 (d, *J* = 7.1 Hz, 3H); ESI-MS m/z = 268. 24 $[M + H]^+$. HPLC tR = 7.41 min, > 97 % purity (λ = 270 nm).

(R/S)-4-benzyl-1-(3-naphthalen-2-yl-butyl)piperidine ***[(R/S)-10c]****:* Yield: 56%, yellow oil; IR (cm^{-1}): 3025, 2924, 2508, 1631, 1602, 1542, 1496, 1453; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.79-7.77 (t, 3H), 7.59 (s, 1H), 7.45-7.42 (m, 2H), 7.32 (m, 1H), 7.26-7.23 (m, 2H), 7.19-7.17 (m, 1H), 7.08 (m, 2H), 3.30 (br. d, 2H), 2.93-2.86 (m, 1H), 2.78-2.73 (m, 1H), 2.55 (d, *J* = 7.0 Hz, 2H), 2.48-2.43 (m, 1H), 2.27-2.13 (m, 4H), 1.82-1.71 (m, 4H), 1.61-1.59 (m, 1H), 1.36 (d, *J* = 6.9 Hz, 3H); ESI-MS m/z = 358.62 $[M + H]^+$. HPLC tR = 11.12 min, > 95 % purity (λ = 270 nm).

(R/S)-4-(3-naphthalen-2-yl-butyl)morpholine ***[(R/S)-10d]****:* Yield: 80%, yellow oil; IR (cm^{-1}): 3053-3026, 2954, 2923-2806, 1599, 1485, 1448, 1115, 836, 763, 732, 696; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7,78-7,81 (m, 3H), 7.61 (s, 1H), 7.48-7.40 (m, 2H), 7.35 (dd, 1H), 3.71 (t, 4H), 2.93 (m, *J* = 7.1 Hz, 1H), 2.40 (br. s, 4H), 2.17-2.34 (m, 2H), 1.93-1.84 (m, 2H), 1.35 (d, *J* = 7.0 Hz, 3H); ESI-MS m/z = 270.13 $[M + H]^+$. HPLC tR = 6.88 min, > 95 % purity (λ = 270 nm).

(R/S)-N,N-dimethyl-[3-(4-methoxy-phenyl)-butyl]-amine ***[(R/S)-11a]****:* Yield: 98%, white solid; mp: 194-195 °C; IR (cm^{-1}): 2951b, 2599, 2362, 2350, 1681, 1514, 1238, 1173, 1031, 828, 668; ^{1}H-NMR (400 MHz) (CDOD) δ (ppm): 7.18 (d, *J* = 8.7 Hz, 2H), 6.9 (d, *J* = 8.7 Hz, 2H), 3.78 (s, 3H), 3.15-3.09 (m, 1H), 2.83 (s, 6H), 2.84-2.76 (m, 1H), 2.02-1.94 (m, 2H) 1.31 (d, *J* = 7.34 Hz, 3H); ESI-MS m/z = 208.15 $[M + H]^+$, HPLC tR = 4.51 min, > 95 % purity (λ = 270 nm).

(R/S)-1-[3-(4-methoxy-phenyl)-butyl]piperidine ***[(R/S)-11b]*****:** Yield: 58%, yellow oil; IR (cm^{-1}): 3030-2994, 2930, 2852-2762, 1611, 1511, 1245, 1176, 1089, 1036, 1010, 827, 790; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.10 (d, *J* = 8.7 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 3.79 (s, 3H), 2.73-2.62 (sextuplet, *J* = 7.1 Hz, 1H), 2.39-2.21 (m, 5H), 2.16-2.09 (m, 1H), 1.78-1.73 (q, *J* = 7.7 Hz, 2H), 1.59-1.51 (m, 4H), 1.41 (m, 2H), 1.22 (d, *J* = 7.7 Hz, 3H); ESI-MS m/z = 248.12 $[M + H]^+$. HPLC tR = 7.00 min, > 95 % purity (λ =270 nm).

(R/S)-4-benzyl-1-[3-(4-methoxy-phenyl)-butyl]piperidine ***[(R/S)-11c]****:* Yield: 76%, yellow oil; IR (cm^{-1}): 3060-3024, 2916, 2833-2766,1609,1510, 1453,1243, 1176, 1036, 827, 744, 698; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.23-7.20 (m, 2H), 7.18-7.10 (m, 3H), 7.09 (d, *J* = 8.7 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 3.78 (s, 3H), 2.85 (br. d, 2H), 2.65-2.52 (sextuplet, *J* = 7.1 Hz, 1H), 2.51 (d, *J* = 7.1 Hz, 2H), 2.26-2.09 (m, 2H), 1.82-1.63 (m, 4H), 1.62-1.56 (m, 2H), 1.47-1.41 (m, 1H), 1.34-1.27 (m, 2H), 1.21 (d, *J* = 7.7 Hz, 3H); ESI-MS m/z = 338.22 $[M + H]^+$. HPLC tR = 10.37 min, > 96 % purity (λ = 270 nm).

(R/S)-4-[3-(4-methoxy-phenyl)-butyl]morpholine ***[(R/S)-11d]****:* Yield: 78%, yellow oil; IR (cm^{-1}): 3030-2993, 2954, 2852-2806, 1611, 1512, 1456, 1245, 1116, 1035, 829; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.12 (d, *J* = 8.7 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 2H), 3.81 (s, 3H), 3.72 (t, 4H), 2.72 (sextuplet, *J* = 7.1 Hz, 1H), 2.41 (br. s, 4H), 2.33-2.16 (m, 2H), 1.79-1.70 (m, 2H), 1.25 (d, *J* = 7.0 Hz, 3H); ESI-MS m/z = 250.45 $[M + H]^+$. HPLC tR = 6.93 min, > 95% purity (λ = 276 nm).

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(R/S)-N,N-dimethyl-[3-(3-methoxy-phenyl)]amine ***[(R/S)-12a]****:* Yield: 72%, white solid; mp: 130-131 °C; IR (cm^{-1}): 3169, 2350, 2326, 1771, 1696, 1484, 1245, 1013, 860, 795, 701; ^{1}H-NMR (400 MHz) (CDOD) δ (ppm): 7.29-7.23 (t, 1H), 6.82-6.75 (m, 3H), 3.80 (s, 3H), 3.20-3.11 (m, 1H), 2.84 (s, 6H), 2.84-2.75 (m, 1H), 2.12-2.00 (m, 2H), 1.33 (d, *J* = 6,85 Hz, 3H); ESI-MS m/z = 208.22 $[M + H]^+$. HPLC tR = 3.96 min, > 99 % purity (λ = 270 nm).

(R/S)-1-[3-(3-methoxy-phenyl)-butyl]piperidine ***[(R/S)-12b]****:* Yield: 77%, yellow oil; IR (cm^{-1}): 3027-2995, 2930, 2852-2736, 1599, 1583, 1486, 1453, 1437, 1257, 1157, 1042, 871, 776, 700; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.21 (t, *J* = 7.0 Hz, 1H), 6.78 (d, *J* = 7.6 Hz, 1H), 6.74-6.70 (m, 2H), 3.80 (s, 3H), 2.68 (m, *J* = 7.1 Hz, 1H), 2.33 (br. m, 5H), 2.29-2.11 (m, 1H), 1.84-1.71 (m, 2H), 1.59-153 (m, 4H), 1.52-1.41 (m, 2H), 1.24 (d, *J* = 6.9 Hz, 3H); ESI-MS m/z = 248.04 $[M + H]^+$. HPLC tR = 6.16 min, > 98% purity (λ = 270 nm).

(R/S)-4-benzyl-1-[3-(3-methoxy-phenyl)-butyl]piperidine ***[(R/S)-12c]****:* Yield: 52%, colorless oil; IR (cm^{-1}): 3082-3024, 2916, 2845-2766,1599, 1583, 1485,1452, 1436, 1258, 1044, 776, 744, 698; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.35-7.25 (m, 2H), 7.24-7.14 (m, 4H), 6.80 (d, *J* = 7.7 Hz, 1H), 6.78-6.73 (m, 2H), 3.82 (s, 3H), 2.89 (br. d, 2H), 2.71 (sextuplet, *J* = 6.9 Hz, 1H), 2.54 (d, 2H), 2.36-2.18 (m, 2H), 1.88-1.76 (br. m, 4H), 1.62 (br. d, 2H), 1.57-1.46 (m, $N(CH_2CH_2)_2C\underline{H}$, 1H), 1.38-1.28 (m, 2H), 1.26 (d, *J* = 6.9 Hz, 3H); ESI-MS m/z = 338.36 $[M + H]^+$. HPLC tR = 8.21 min, > 96 % purity (λ = 270).

(R/S)-4-[3-(3-methoxy-phenyl)-butyl]morpholine ***[(R/S)-12d]****:* Yield: 43%, yellow oil; IR (cm^{-1}): 3050-3024, 2954, 2852-2806, 1607, 1598, 1583, 1486, 1454, 1259, 1116, 1043, 867, 778, 700; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.21 (t, *J* = 7.7 Hz, 1H), 6.78 (d, *J* = 7.7 Hz, 1H), 6.74-6.72 (m, 2H), 3.80 (s, 3H), 3.70 (t, 4H), 2.72 (m, *J* = 7.1 Hz, 1H), 2.39 (br. s, 4H), 2.31-2.16 (m, 2H), 1.79-1.72 (m, 2H), 1.25 (d, *J* = 7.0 Hz, 3H); ESI-MS m/z = 250.16 $[M + H]^+$. HPLC tR = 6.25 min, > 95 % purity, (λ = 270 nm).

(R/S)-N,N-dimethyl-(3-phenyl-butyl)-amine ***[(R/S)-13a]****:* Yield: 95%, white solid; mp: 220-222 °C; IR (cm^{-1}): 2957, 2462, 2362, 2313, 1471, 1315, 1173, 1017, 959, 764, 708; ^{1}H-NMR (500 MHz) (CDOD) δ (ppm): 7.37-7.25 (m, 2H), 7.25-7.12 (m, 3H), 3.21-3.10 (m, 1H), 2.83 (s, 6H), 2.79-2.75 (m, 1H), 2,03-1.95 (m, 2H), 1,32 (d, *J* = 6.8 Hz, 3H); ESI-MS m/z = 178.23 $[M + H]^+$. HPLC tR = 17.12 min, > 95 % purity (λ = 276 nm).

(R/S)-1-(3-phenyl-butyl)piperidine ***[(R/S)-13b]*****:** Yield: 58%, yellow oil; IR (cm^{-1}): 3083-3026, 2929, 2852-2762, 1602, 1493, 1451, 1154, 1120, 759, 698; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.29 (t, *J* = 8.1 Hz, 2H), 7.19-7.16 (m, 3H), 2.73-2.68 (sextuplet, *J* = 7.1 Hz, 1H), 2.36-2.26 (m, 5H), 2.19-2.12 (m, 1H), 1.84-1.78 (m, 2H), 1.61-1.56 (m, 2H), 1.41 (m, 2H), 1.25 (d, *J* = 6.9 Hz, 3H); ESI-MS m/z = 218.17 $[M + H]^+$. HPLC tR = 9.27 min, > 95 % purity (λ = 250 nm).

(R/S)-4-benzyl-1-(3-phenyl-butyl)piperidine ***[(R/S)-13c]****:* Yield: 47%, yellow oil; IR (cm^{-1}): 3682, 3019, 2929, 2856, 2434, 2400, 1230; ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.36-7.25 (m, 4H), 7.24-7.13 (m, 6H), 2.86 (br. d, 2H), 2.71 (sextuplet, *J* = 7.0 Hz 1H), 2.52 (d, *J* = 7.1 Hz, 2H), 2.34-2.21 (m, 1H), 2.20-2.11 (m, 1H), 1.89-1.72 (m, 4H), 1.61 (br. d, 2H), 1.58-1.42 (m, 1H), 1.39-1.30 (m, 2H), 1.28 (d, *J* = 7.2 Hz, 3H). ESI-MS m/z = 308.19 $[M + H]^+$. HPLC tR = 8.40 min, > 98 % purity (λ = 270 nm).

(R/S)-4-(3-phenylbutyl)morpholine ***[(R/S)-13d]****:* Yield: yellow oil; IR (cm^{-1}): 2972, 2857, 1602, 1492, 1445, 1370, 1265, 1116, 914, 860. ^{1}H-NMR (400 MHz) ($CDCl_3$) δ (ppm): 7.40-7.35 (m, 2H), 7.30-7.22 (m, 1H), 7.18-7.10 (m, 2H), 3.66-3.62 (m, 4H), 2.73 (m, 1H), 2.54-2.49 (m, 2H), 2.37-2.24 (m, 2H), 2.22-2.14 (m, 2H), 1.82-1.71 (m, 2H), 1.27 (d, *J* = 7.0 Hz, 3H). ESI-MS m/z = 220.42 $[M + H]^+$. HPLC tR = 8.72 min, > 96 % purity (λ = 250 nm).

5.2 Binding assays

The affinities of compounds 10-13 a-d towards S1R and S2R were evaluated by radioligand receptor binding studies.

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The assay for S1R is based on the use of membrane from guinea pig cerebral cortex, which represents a receptor source in the presence of a potent and selective S1R radioligand (i.e. [^{3}H]-(+)-pentazocine). Nonspecific binding values were determined using non-radiolabeled (+)-pentazocine and haloperidol in large excess.

Instead, in the case of S2R, we used the membrane of rat liver as receptor source. This test was performed using a nonselective radioligand ([^{3}H]-DTG), since no S2R selective radioligand are commercially available. Moreover, it is important to mask the S1R: for this reason, an excess of non-tritiated (+)pentazocine was added to the assay solution. In order to determine nonspecific binding, a high concentration of non-tritiated DTG was used.

5.2.1 Materials

Guinea pig brains for the S1R binding assays were commercially available (Harlan–Winkelmann, Borchen, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Vortexer: Vortex Genie 2 (Thermo Fisher Scientific, Langenselbold, Germany). Harvester: MicroBeta FilterMate-96 Harvester. Filter: Printed Filtermat Type A and B. Scintillator: Meltilex (Type A or B) solid-state scintillator. Scintillation analyzer: MicroBeta Trilux (all PerkinElmer LAS, Rodgau-Jügesheim, Germany). Chemicals and reagents were purchased from various commercial sources and were of analytical grade.

Preparation of membrane homogenates from guinea pig brain cortex: Five guinea pig brains were homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in six volumes of cold 0.32m sucrose. The suspension was centrifuged at 1200 g for 10 min at 4°C. The supernatant was separated and centrifuged at 23500 *g* for 20 min at 4°C. The pellet was resuspended in 5–6 volumes of buffer (50 mm Tris, pH 7.4) and centrifuged again at 23500 *g* (20 min, 4 8C). This procedure was repeated twice. The final pellet was resuspended in 5–6 volumes of buffer and frozen (-80°C) in 1.5 mL portions containing ~1.5 (mg protein)mL^{-1}.

5.2.2

Protein determination: The protein concentration was determined by the method of Bradford[43] modified by Stoscheck[44]. The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL EtOH (95% v/v). Deionized H_2O (10 mL) and phosphoric acid (85% w/v, 5 mL) were added to this solution, and the mixture was stirred and filled to a total volume of 50 mL with deionized water. Calibration was carried out using bovine serum albumin as a standard in nine concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 4.0 mg mL^{-1}). In a 96-well standard multiplate, 10 mL of the calibration solution or 10 mL of the membrane receptor preparation were mixed with 190 mL of the Bradford solution. After 5 min, the UV absorption of the protein–dye complex at l=595 nm was measured with a plate reader (Tecan Genios, Tecan, Crailsheim, Germany).

5.2.3 General protocol for binding assays

The test compound solutions were prepared by dissolving ~10 mmol (usually 2–4 mg) of test compound in DMSO so that a 10 µM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5% aqueous polyethylenimine solution for 2 hours at RT before use. All binding experiments were carried out in duplicate in 96-well multiplates. The concentrations given are the final concentrations in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL test compound solution at various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}M), 50 µL of corresponding radioligand solution, and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the

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multiplates were shaken at a speed of 500–600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration each well was washed five times with 300 mL of water. Subsequently, the filtermats were dried at 95°C. The solid scintillator was melted on the dried filtermats at 95°C for 5 min. After solidifying of the scintillator at RT, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [^{3}H]-counting protocol. The overall counting efficiency was 20%. The IC50 values were calculated with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) by nonlinear regression analysis. The IC50 values were subsequently transformed into Ki values using the equation of Cheng and Prusoff.[45] The Ki values are given as mean value ±SEM from three independent experiments.

5.2.4 S1R binding assay

The assay was performed with the radioligand [^{3}H](+)-pentazocine (22.0 Ci mmol^{-1}; PerkinElmer). The thawed membrane preparation of guinea pig brain cortex (~100 mg protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H](+)-pentazocine, and Tris buffer (50 mM, pH 7.4) at 37°C. The nonspecific binding was determined with 10 mM unlabeled (+)-pentazocine. The Kd value of (+)-pentazocine is 2.9 nM.

5.2.5 S2R binding assay

The assay was performed using 150 µg of rat liver homogenate were incubated for 120 min at room temperature with 3 nM [^{3}H]-DTG (Perkin–Elmer, specific activity 58.1 Ci mmol^{-1}) in 50 mM Tris–HCl, pH 8.0, 0.5 mL final volume. (+)-pentazocine (100 nM) and haloperidol (10 µM) were used to mask S1R and to define non-specific binding, respectively.

6. QSAR Modelling

We generated 3D structures of 75 arylalkylamine derivatives by using MOE dedicated tools5. Before calculating descriptors, we prepared the structures by using its "wash" function to protonate them at physiological pH. Energy minimization followed using MMFF94x and applying default settings[46].

We divided our dataset into training and test set: the training set contains 61 derivatives, that were already published in literature and other in-house compounds (unpublished data). We generated the QSAR models based on the training set. We calculated and scaled all available 2D and 3D descriptors in MOE: the most important 7 descriptors were selected out of 338 by considering their correlation to the assay data (**Supplemental Material Table1**). Affinity data were converted to pKi (-log$_{10}$Ki) values in order to normalize the range of data and perform a linear regression. More negative pKi values indicate higher Ki values and, hence, lower affinity. QSAR models were generated by Partial Least Square analysis with a limit of 3 principal components and validated by cross validation.

QSAR models have been validated internally by Leave-One-Out (LOO) cross validation and externally by testing our new 14 derivatives.

The model for receptor S1R has a correlation coefficient R^2 0.64 and root mean square error RMSE 0.64; cross-validated correlation coefficient (Q^2) is 0.55, with RMSE of 0.72, indicating that the prediction is reliable. We modeled QSARs also for S2R receptor, by using the same procedure and descriptors (correlation coefficient R^2 0.58, root mean square error RMSE 0.44, cross-validated correlation coefficient Q^2 0.48, cross-validated RMSE 0.49).

pK_{iS1R} = - 1.44302 + 0.23255 * BCUT_SLOGP_3 - 0.33249 * a_don + 0.27685 * E_sol + 0.20083 * b_rotN - 0.13502 * glob - 0.03737 * dipole - 0.46869* AM1_IP

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pK_{iS2R} = - 2.23985 + 0.27149 * BCUT_SLOGP_3 - 0. 20203 * a_don + 0.12047 * E_sol + 0.14450 * b_rotN - 0.11578 * glob - 0.24609 * dipole - 0.02238 * AM1_IP

The equation that describes the linear correlation indicates that the descriptors contribute to the binding affinity with positive or negative coefficients. Descriptors, that contribute to the predicted pKi values with a negative coefficient, decrease the binding affinity. On the opposite, descriptors, that contribute to the predicted pKi values with a positive coefficient, increase it.

To estimate whether selected descriptors are not inter-correlated, we generated a correlation matrix by using the respective MOE tool: we found that the absolute correlation is lower than 0.5 for all descriptors, but E_sol that is orthogonal to AM1_IP and dipole descriptors with correlation of 0.73 and 0.64 respectively. However, AM1_IP and dipole do not exceed the threshold of 0.5 (correlation 0.22).

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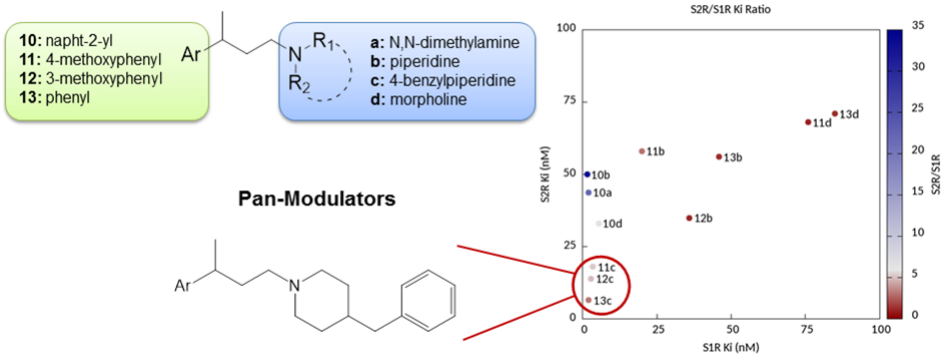

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