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A vHTS approach for the identification of β-adrenoceptor ligands

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ABSTRACT

Using a vHTS based on a pharmacophore alignment on known β3-adrenoceptor ligands, a set of intriguing β-adrenoceptor ligands was identified, optimization of which resulted in a selective and potent human β2-AR antagonist.

Many ligands for β-adrenergic receptors are currently used to treat a range of disorders. These marketed drugs still address purposely only the human β1- and β2-adrenergic receptor subtypes (hβ1- and hβ2-AR). This is due to the fact that a third subtype, hβ3-AR, was not discovered and cloned until the late 80s[1] and clinical trials for the use of hβ3-agonists to treat obesity and diabetes—which represented the primary focus back then—were rather disappointing.[2–4] β-Blockers, most ideally β1-selective, are effective in the treatment of angina pectoris, heart failure and hypertension.[2,5] The inhalation of β2-adrenoceptor-selective agonists has been established as therapy of asthma and other bronchospastic conditions for some time.[2,6] Furthermore, systemic β2-agonists are used as tocolytics in preterm labor.[6–8] With the hβ3-AR being predominantly located in adipose tissue but also playing a crucial role in the gastrointestinal tract and the bladder smooth muscle, stimulation of this receptor represents a potential therapeutic approach to treat irritable bowel syndrome or urinary incontinence[2,9,10]—and still obesity, with a phase II clinical trial in this indication ongoing for LY-377604 in combination with Sibutramine.[11] Upon the identification of the β3-AR in near-term human myometrium, agonists of this receptor have also been discussed as rescue therapy for the treatment of preterm labor.[7]

Focusing primarily on the identification of new hβ3-adrenoceptor ligands, a pharmacophore alignment was chosen for a virtual high throughput screening (vHTS) approach on hβ3-AR. At the given point in time, only the crystal structure of the bovine rhodopsin receptor was available as a representative of the target class of GPCRs,[12] with only around 40% sequence homology as compared to hβ3-AR. This changed not before 2007 when crystal structures were resolved for the human β2-receptor[13] and, shortly afterwards, for the turkey β1-receptor.[14] Consequently, a direct docking approach on hβ3-AR using a homology model was not selected as method of choice back then. For an alignment approach, five different literature-known hβ3-AR agonists were selected as templates based on their advancement in clinical development, their activity on β3-receptors and/or for structural reasons (Fig. 1): two phase II candidates at the given point in time (KUC-7322 and N-5984, displaying EC_{50} values in the lower or even sub-nanomolar range in different functional β3-AR assays),[9,15] or structurally intriguing compounds **1** (CP-331,679, full agonist, EC_{50}(hβ3) = 300 nM),[3,4] **2** (sub-nanomolar agonist on β3-AR)[4] and the non-subtype-selective isoquinoline **3** (one-digit nanomolar EC_{50} values on all three human β-adrenergic subtypes).[16]

Each of these templates was used for a vHTS run of a virtual library comprising 4.6 Mio commercially available compounds using the 4SCan® technology.[17] Top scoring 5000 molecules of each run were combined and filtered by molecular weight (<600 g/mol), TPSA (<200 Å^2), number of rotatable bonds (<20) and violation of Lipinski rules (max 1). Out of the resulting list of approximately 800 compounds of interest, about 210 were selected driven by chemical diversity for biological screening in a binding assay on hβ3-AR.[18] Three compounds were identified with K_i values <20 μM, based on which a 'back-screening' was initiated: another 420 compounds were selected for biological evaluation according to their structural similarity with the initial hit molecules. This

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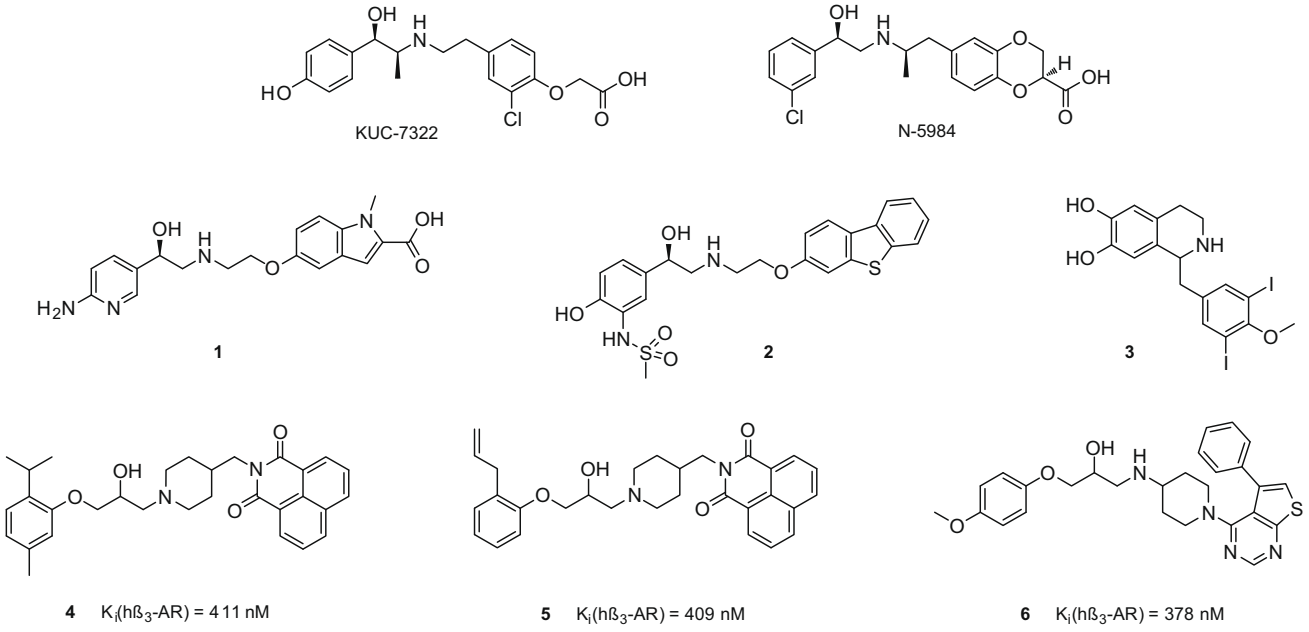


Figure 1. Templates chosen for a pharmacophore alignment and vHTS hits **4**–**6**.

approach yielded compounds displaying even better affinity, now with sub-micromolar K_i values, the best of which are depicted in Figure 1 (compounds **4**–**6**).[19]

As an alignment procedure is based on the generation of a 'pseudo-receptor' out of the template pharmacophore, application of this method allows only for matching interaction possibilities of the template used but does not provide any information about the binding site itself. Subtle changes within a given structural scaffold can result in drastic effects on selectivity and/or functional activity, potentially covering the whole range from an agonist to an inverse agonist, with such variations possibly not lying within the pseudo-receptor area. Thus, such a modeling approach is very useful for the identification of new structural entities as ligands for a target group of high homology (e.g., the β-adrenoceptors), a prediction of selectivity and/or functional activity, however, can not necessarily be expected—even though the selection of templates might bias the outcome. Consequently, a hit validation had to follow, including the performance of binding and functional assays for all three β-AR subtypes.[20,21]

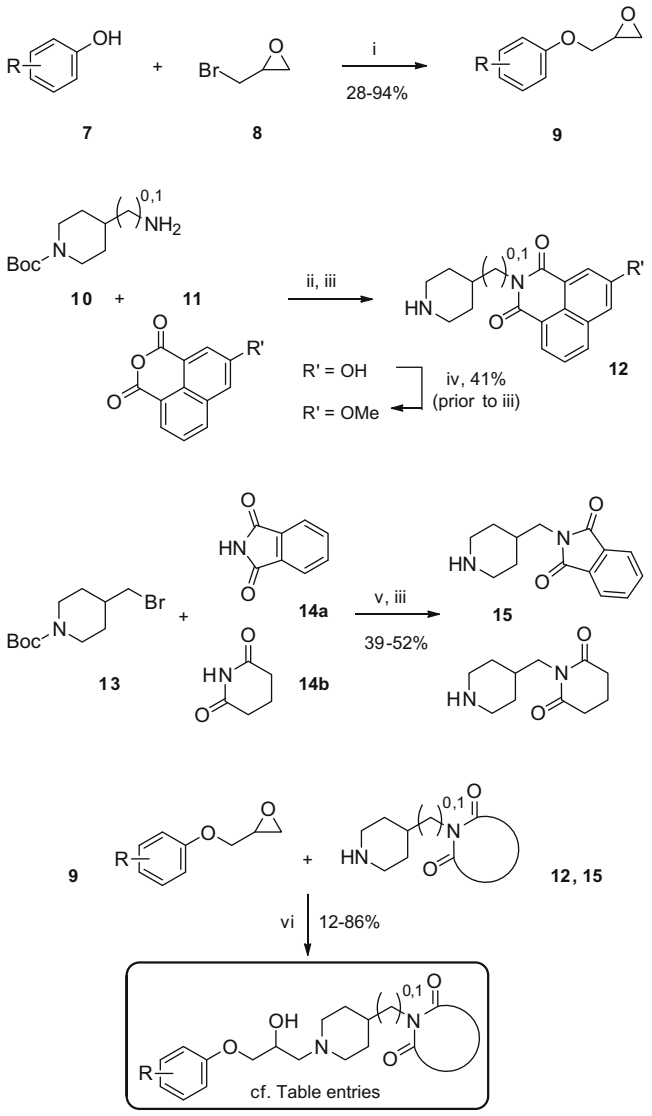
For naphthalimide derivatives **4** and **5**, sub-micromolar affinities on hβ3-AR as attained from the initial screening campaign could not be confirmed. Both compounds displayed K_i values slightly below 2 μM (Table 1). However, affinity for hβ2-AR was quite good with K_i values of 80 nM for both compounds, one displaying already some selectivity over hβ1-AR (compound **4**, by factor 12). Compound **5** exhibited similar affinities for hβ1-AR and hß2-AR. With regard to functional activity, compound **4** possessed antagonistic activity on all three β-AR subtypes, the strongest effect again on hβ2-AR with an IC_{50} value of 377 nM. As mentioned above, slight structural changes might already alter functional activity significantly. This was confirmed by the structurally very similar naphthalimide **5**, which displayed weak partial agonism on hβ3-AR with an intrinsic activity of 65% as compared to the standard isoproterenol. For thienopyrimidine **6**, the sub-micromolar affinity for hβ3-AR as detected in the initial screening was confirmed. It already displayed a slight selectivity for hβ3-AR over hβ2-AR based on binding data and proved to be a partial agonist on hβ3-AR as well, but now on an even better activity level with an EC_{50} value of 120 nM. These results validated an intriguing starting point for medicinal chemistry. SAR evaluations for the naphthalimides will be described herein, whereas those for the thienopyrimidines will be disclosed in due course.[22]

Table 1
Binding and functional data on β-AR subtypes for hit molecules out of vHTS

Compd	Binding assay, K_i [nM]			Functional assay, EC_{50}/IC_{50} [nM] (IA)[a]		
	hβ1-AR	hβ2-AR	hβ3-AR	hβ1-AR	hβ2-AR	hβ3-AR
4	1000	80	1630	IC_{50} = 1375	IC_{50} = 377	IC_{50} = 1474
5	80	80	1850	nd	nd	EC_{50} = 2400 (0.65)
6	>10,000	700	460	nd	nd	EC_{50} = 120 (0.6)

[a] EC_{50} indicates agonistic, IC_{50} indicates antagonistic effects; IA = intrinsic activity, relative to isoproterenol (IA = 1.0); nd = not determined.

For a systematic evaluation of structural effects on selectivity and functional activity within the naphthalimide series, the aryloxypropanolamine array was kept constant and the substitution pattern of the aryloxy portion as well as the aminopiperidine spacer unit were subjected to alterations. Synthetically, such derivatives were obtained as follows: required 2-(aryloxymethyl)oxiranes **9** were prepared by O-alkylation of phenols **7** using epibromohydrine (**8**).[23] 4-Piperidinyl and piperidin-4-ylmethyl-naphthalimides **12** were attained from the corresponding primary amine **10** and a 1,8-naphthalic anhydride **11**. For smaller imide building blocks **15**, deprotonation of phthalimide (**14a**) or glutarimide (**14b**) and subsequent N-alkylation with 4-(bromomethyl)piperidine **13** proved to be a better route as compared to transformation of cyclic anhydrides with the corresponding amines. Finally, reaction of oxiranes **9** with piperidines **12** or **15** occurred in isopropanol at 60–80 °C.[24] This procedure was likewise effective with morpholine (cf. compound **35**, Table 4), 1-methylpiperidin-4-amine (cf. compounds **36** and **37**), 4-benzylpiperidines (cf. compounds **41**–**43**, Table 5) and 4-benzoylpiperidines (cf. compounds **44**–**46**). DIEA had to be added only if the respective amine was used as its hydrochloride salt. For compounds bearing a 4-hydroxyaryloxy moiety (R = OH, Scheme 1), the sequence was realized with an *O*-Bz protective group, which was saponified in the final product using 3 N aq NaOH in dioxane (100 °C, 2 h). Same

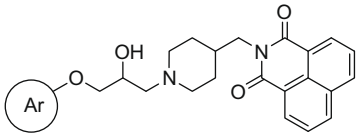


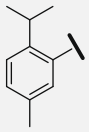
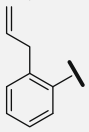
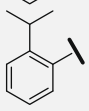
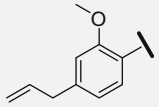
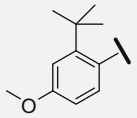
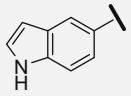
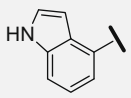
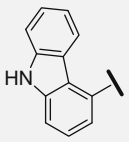
Scheme 1. Reagents and conditions: (i) K_2CO_3, butanone, 80 °C, 24–48 h; (ii) DMF, 160 °C, 1–3 h (R′ = H, OH), 64–80%; (iii) HCl in dioxane (4.0 M), 0 °C, 2 h, quant.; (iv) conducted in between steps ii and iii: (a) NaH, DMF, 0 °C to rt, 30 min; (b) MeI, 0 °C to rt, 30 min; (v) (a) NaH, DMF, 0 °C to rt, 30 min; (b) **13**, 80–100 °C, 10–20 h; (vi) depending on solubility: *i*PrOH or *i*PrOH/DMSO (up to 1:1), DIEA (optional), 60–80 °C, 2–8 h; yields are not optimized.

conditions were applied for the conversion of a benzoic ester derivative into the corresponding acid (R = COOMe to R = COOH, Table 2).

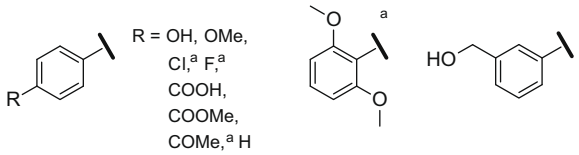
Within the set of differently substituted aryloxy units, a few patterns were also realized known from other β-adrenoceptor ligands: *p*-hydroxyphenyloxy (Ritodrine, β2-agonist; KUL-7211, β2/β3-agonist),[9] carbazolyloxy (Carvedilol, β1/β2/β3-antagonist; Carazolol, β1/β2-antagonist; LY-377604, β3-agonist), 4-indolyloxy (pindolol, β1/β2-antagonist), 2-allylphenyloxy (Alprenolol, β1/β2-antagonist; SR58894, β3-antagonist; already present in hit molecule **5**)[2,9,25] and 4-allyl-2-methoxyphenyloxy (β1/β2-antagonist).[2] As can be seen from Table 2, none of the aryloxy variations resulted in a shift of selectivity towards hβ3-AR. The best K_i on this receptor was observed for the carbazolyl derivative **21** with 225 nM, which, however, also experienced a significant increase of affinity for hβ1- and hβ2-AR, now being in the one-digit nanomolar region. Generally, these naphthalimide derivatives displayed best affinities for hβ2-AR. The only compound with some selectivity for hβ1-AR within a decent affinity range was 4-indolyl derivative **20** with a K_i of 27 nM. 2-Allyl and 2-isopropyl derivatives **5** and **16** displayed similar affinity for hβ1- and hβ2-AR. Incorporation of an additional methyl group, however, resulted in some hβ2-AR selectivity (compound **4** vs **16**, factor 12 towards hβ1-AR, factor 20 towards hβ3-AR). Attachment of oxygen-based polar groups was generally detrimental to affinity for either receptor (compound **17** and summary of inactive derivatives, Table 2; to a lesser extent also **18**). Not even respective aromatics deduced from reference ligands were able to

Table 2
Binding data of naphthalimide derivatives on the three β-AR subtypes: variations of the aryloxy moiety[19]



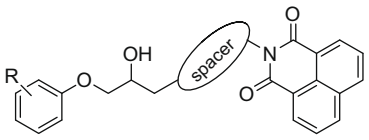
Compd	Ar	Binding assay, K_i [nM]		
		hβ1-AR	hβ2-AR	hβ3-AR
4[a]		1000	80	1630
5[a]		80	80	1850
16		250	230	1030
17[a]		1360	3290	1180
18[a]		530	300	3380
19		4530	1260	8500
20		27	120	1000
21		7	3	225

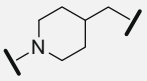
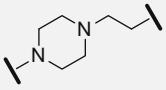
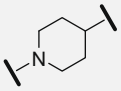
inactive (>10 μM) at hβ3-AR:



[a] Compound from a commercial source.

Table 3
Binding data of naphthalimide derivatives on the three β-AR subtypes: variations of the spacer unit[19]



Compd	Spacer	R	Binding assay, K_i [nM]		
			hβ1-AR	hβ2-AR	hβ3-AR
4		2-*i*Pr-5-Me	1000	80	1630
5		2-Allyl	80	80	1850
16		2-*i*Pr	250	230	1030
22[a]		2-*i*Pr-5-Me	Inactive	110	>10,000
23[a]		2-Allyl	293	150	3100
24[a]		2-*i*Pr	740	70	1480
25		2-Allyl	1050	40	2340
26		2-*i*Pr	240	13	3820

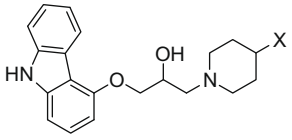
[a] Compound from a commercial source.

give optimized affinities (*p*-hydroxyphenoxy, and 4-allyl-2-methoxyphenyloxy, compound **17**).

Next, the influence of spacer length in between the propanolamine and the naphthalimide unit on affinity and selectivity was investigated (Table 3). In all three variants, the spacer was based on a six-membered heterocyclic ring (piperidine or piperazine), and the alkyl chain $(CH_2)_n$ attached to the cyclic amine was varied from $n = 0$ to $n = 2$. In order to allow for a thorough SAR conclusion, these variants were incorporated into three different sub-series defined by the substitution pattern at the aryloxy portion (2-isopropyl-5-methyl vs 2-allyl vs 2-isopropyl).

For all three subsets of aryloxy variants, extension or diminution of the spacer unit $(CH_2)_n$ by one methylene unit (which was accompanied by a piperidine exchange to piperazine for the extended $n = 2$ version, though) enhanced selectivity for hβ2-AR. This effect was caused by a general decrease of affinity for β3-AR and—with only one exemption (compound **26** vs **16**)—for hβ1-AR. With-

Table 5
Binding data of carbazol-4-yloxy derivatives on the three β-AR subtypes: variations of the imide part[19]



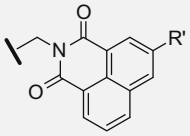
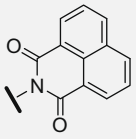
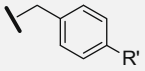
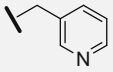
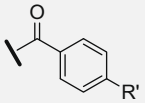
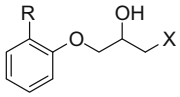
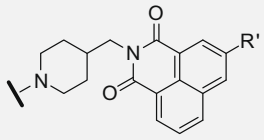
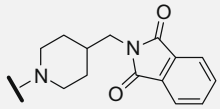
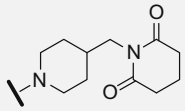
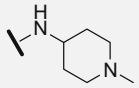
Compd	X	R′	Binding assay, K_i [nM]		
			hβ1-AR	hβ2-AR	hβ3-AR
21		H	7	3	225
38		OH	250	37	590
39		OMe	0.1	8	410
40			76	57	98
41		H	14	0.5	230
42		OMe	3.4	0.2	455
43			1.0	0.1	72
44		H	16	0.2	230
45		OMe	13	0.2	9180
46		F	32	0.3	250

Table 4
Binding data of naphthalimide derivatives on the three β-AR subtypes: variations of the imide part[19]



Compd	X	R	R′	Binding assay, K_i [nM]		
				hβ1-AR	hβ2-AR	hβ3-AR
5		Allyl	H	80	80	1850
27		Allyl	OH	170	135	2200
28		Allyl	OMe	300	78	1510
16		*i*Pr	H	250	230	1030
29		*i*Pr	OMe	209	38	698
30		Allyl		190	50	3400
31		*i*Pr		720	17	1480
32		Allyl		440	80	11,000
33		*i*Pr		245	9	2900
34[a]		Allyl		114	7	4960
35		*i*Pr		12	1.2	1770
36		Allyl		260	2.4	6225
37		*i*Pr		89	17	1060

[a] Compound from a commercial source.

in the shortened piperidine series ($n = 0$; compounds **25** and **26**), affinity for hβ2-AR was simultaneously increased, resulting in a good K_i of 13 nM for the 2-*i*Pr derivative **26**.

When removing some bulk from the naphthalimide portion, which is known to potentially cause DNA intercalation[26] and was thus sought to be avoided, affinity for hβ3-AR was further decreased for phthalimides to glutarimides within both, the allyl and isopropyl series (compounds **5** vs **30** vs **32** and **16** vs **31** vs **33**; Table 4). For both of these series, a clearly optimized hβ2-AR selectivity was achieved: for allyl derivatives, affinity for hβ1-AR decreased as well from naphthalimides over phthalimides to the glutarimides accompanied by a nearly constant affinity for hβ2-AR; for isopropyl compounds, the affinity for hβ1-AR remained within the same range along with an increase of affinity for hβ2-AR. Leaving out any imide array and thus rather mimicking the Alprenolol scaffold (compounds **34** and especially **36**), ligands with good affinities for hβ2-AR were attained with K_i values of 7 and 2.4 nM, respectively—and even with good selectivity by a factor of 110 over hβ1- and above 2000 over hβ3-AR (compound **36**). The corresponding isopropyl derivatives **35** and **37** possessed an increased affinity for hβ1- and hβ3-AR as compared to the 2-allyl variants **34** and **36**, thus resulting in an overall decreased selectivity for hβ2-AR. However, an even better K_i of 1.2 nM was detected for compound **35** at the latter receptor.

All variations described so far—at the aryloxy unit, at the spacer, and fragmentations of the naphthalimide unit—were leading to an optimization of selectivity and affinity for hβ2-AR. Therefore, an attempt was made to gain some affinity for hβ3-AR by performing a Comparative Molecular Similarity Indices Analysis (CoMSIA)[27] based on those compounds elaborated around Table 2 combined with several thienopyrimidine derivatives around hit molecule **6**, which proved to be β3-AR selective.[22] A resulting CoMSIA sketch (Fig. 2) suggested the addition of a hydrogen bond acceptor to the 5-position of the naphthalimide to enhance ligand affinity, which was realized by incorporating a hydroxy or methoxy group (R′ in Table 4). Such derivatizations, however, were not able to generate compounds with (significantly) increased affinity for hβ3-AR. Instead, hβ2-AR selectivity was optimized for compounds **28** and **29**, bearing a methoxy group (Table 4), as compared to the parent molecules **5** and **16**.

Finally, the carbazolyl derivative **21** was selected for a set of derivatizations at the naphthalimide site (Table 5), as it was the only compound identified so far to display significant affinity for hβ3-AR—even though still topped by those for hβ1- and hβ2-AR.

Again, as already observed for the 2-allyl- and 2-isopropyl-phenoxy series (Table 4), substitution at C5 of the naphthalimide in order to add a hydrogen bond acceptor was not providing a shift towards hβ3-AR-selective compounds. Instead, a ligand with an excellent affinity for hβ1-AR was attained (compound **39**; $K_i = 0.1$ nM), displaying a decent selectivity over hβ2-AR. Only shortening the spacer unit by one methylene group as realized in compound **40** resulted in a stronger emphasis of affinity for hβ3-AR by reducing affinities for hβ1- and hβ2-AR. The overall outcome was a mediocre K_i(hβ3) of 98 nM and a comparable affinity level for both of the other β-subtypes. A replacement of the naphthalimidomethylene by either benzyl or benzoyl derivatives eventually shifted the focus completely: excellent picomolar affinities were attained on hβ2-AR. By variation of the substitution pattern at the aromatic ring, selectivity over hβ1-AR seems to be tunable, with the *para*-fluoro derivative **46** displaying good selectivity factors of 105 over hβ1-Ar and 830 over hβ3-AR (based on binding data). For this compound **46** and derivative **38**, functional data was acquired (Table 6): both displayed a moderate partial agonistic activity at hβ1-AR, a mediocre full agonistic effect on hβ3-AR and good antagonistic activity at hβ2-AR, with compound **46** giving rise to an IC_{50} of 32 nM. For the 'best' hβ3-AR ligand out of this series, compound **40**, agonistic activity on hβ3-AR was rather poor with an $EC_{50} = 2.8$ μM (IA = 0.9; data not shown).

With a beneficial effect of hβ2-AR antagonists in wound healing having been described recently,[28] compound **46**[29] displayed an intriguing activity profile. Consequently, this substrate was subjected to further biological and physicochemical evaluation. It proved to be completely stable in artificial gastric juice, simulated intestinal fluids and human plasma within a test period of 6 h. Solubility was determined to be above 180 μM in aq. medium buffered at pH 4 and 10 μM at pH 6, membrane permeability in a PAMPA was high. Toxicity in a PBMC viability assay was found at a tolerable level with an $ED_{50} = 9$ μM, and in a HepG2 assay with an $ED_{50} = 19$ μM. A preliminary PK study in male Wistar rats was performed by po administration of the compound (10 mg/kg), resulting in a C_{max} of 290 nM and a halflife $t_{1/2}$ of 7.5 h.

The vHTS approach as realized herein enabled the identification of potent β-adrenoceptor ligands. Subtype selectivity or a well defined cellular functional activity, however, was not necessarily attained into the direction of the ligands used for the pharmacophore alignment: thienopyrimidines like **6** (Fig. 1) showed a certain selectivity for hβ3-AR based on binding data and a partial agonistic effect on this receptor in a cellular assay[22]—as originally envisaged when template ligands were selected—naphthalimides **4** and **5** displayed highest affinity for hβ2-AR. For compound **4**, this affinity was accompanied by a certain selectivity combined with an antagonistic effect on all three β-AR subtypes. Derivative **5** showed similar affinity for hβ1- and hβ2-AR with a weak agonistic effect on hβ3-AR. As mentioned above, a pharmacophore alignment approach can not necessarily be expected to yield a more defined result with regard to selectivity and functional activity, which was clearly reflected in this study. Taken from recent literature, a docking approach based on the crystal structure of the inactive state of the hβ2-AR might not result in ligands with a clearly defined functional activity either.[30] Thus, the simplicity of the pharmacophore alignment in combination with the good affinities attained for the

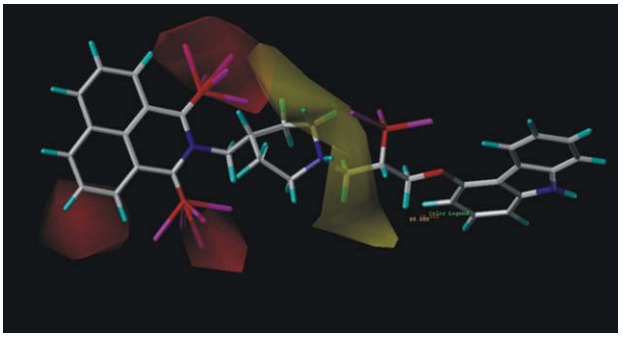


Figure 2. CoMSIA illustration around compound **21**: red area indicates a hydrogen bond acceptor to be desirable within ligand, yellow area indicates a hydrogen bond donor to be desirable within ligand.

Table 6
Binding and functional data for selected β-AR ligands

Compd	Binding assay, K_i [nM]			Functional assay, EC_{50}/IC_{50} [nM] (IA)[a]		
	hβ1-AR	hβ2-AR	hβ3-AR	hβ1-AR	hβ2-AR	hβ3-AR
4	1000	80	1630	$IC_{50} = 1375$	$IC_{50} = 377$	$IC_{50} = 1474$
38	250	37	590	$EC_{50} = 80$ (0.4)	$IC_{50} = 120$	$EC_{50} = 234$ (1.0)
46	32	0.3	250	$EC_{50} = 310$ (0.2)	$IC_{50} = 32$	$EC_{50} = 385$ (1.1)

[a] EC_{50} indicates agonistic, IC_{50} indicates antagonistic effects; IA = intrinsic activity, relative to isoproterenol (IA = 1.0).

original screening hits renders this approach highly valuable. Starting from the naphthalimide-substituted aryloxypropanolamine scaffold of the original hit molecules **4** and **5**, all medicinal chemistry endeavours resulted in a stronger emphasis of affinity for hβ2-AR and optimization of selectivity towards the latter AR subtype. Such efforts led to the identification of the benzoylpiperidine derivative **46**, displaying excellent affinity for hβ2-AR in the picomolar range and an antagonistic activity at hβ2-AR. Its favourable pilot profile of physicochemical and pharmacokinetic parameters suggests further optimization towards application of a hβ2-AR antagonist in for example, wound healing.

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18. Initial screening was performed at MDS, Taiwan, in a radioligand receptor binding assay at a compound concentration of 10 μM (in duplicate). For potential hits (radioligand replacement >20%), a semi-quantitative IC_{50} was determined (six concentrations in duplicate). The assay was based on membrane preparations of HEK-293 cells overexpressing the human β3-AR (B_{max} = 550 fmol/mg protein) using 0.5 nM [^{125}I]-cyanopindolol as radioligand.
19. All aryloxypropanolamine derivatives were tested as racemates.
20. Membrane preparations (CHO-K1 cell line) expressing human β1-, β2- or β3-ARs (B_{max} = 3.78, 1.68 and 47.2 pmol/mg protein, respectively), were purchased from Euroscreen (now Perkin Elmer). Binding assays were performed according to the manufacturer's instructions. The radioligand for all three receptor subtypes was [^{125}I]-cyanopindolol ([^{125}I-CYP) (Amersham) (final concentration of 0.05, 0.05 and 1.5 nM, respectively). K_i values were calculated using the Cheng–Prusoff equation on IC_{50} determinations, which were based on concentration curves using eight concentrations (half-logarithmic) in duplicate.
21. Functional response of cells (agonistic or antagonistic) to the test compounds was tested by measurement of cyclic AMP formation by HTRF® (Homogeneous Time-Resolved Fluorescence) technology (Cisbio International) using a stable cell line CHO-K1 expressing the human recombinant β3-AR (Euroscreen, now Perkin Elmer) according to the manufacturer's instructions. EC_{50} (agonists) and IC_{50} values (antagonists) were determined by dose–response curves based on eight concentrations (logarithmic) determined in quadruplicate in a 96 half-well plate in a final volume of 100 μl. The antagonistic effect was determined by preincubation with a test compound for 10 min followed by agonist stimulation (0.05 nM isoproterenol) for 30 min.
Functional data on hβ1-AR and hβ2-AR were determined at Euroscreen (both, in an agonist and an antagonist assay). EC_{50} and IC_{50} values, respectively, were determined by dose response curves based on eight concentrations (logarithmic) determined in duplicate.
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