Synthesis, Binding Affinity and Structure-Activity Relationships of Novel, Selective and Dual Targeting CCR2 and CCR5 Receptor Antagonists

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Abstract

CCR2 and CCR5 receptors play a key role in the development and progression of several inflammatory, cardiovascular and autoimmune diseases. Therefore, dual targeting of both receptors appeals as a promising strategy for the treatment of such complex, multifactorial disorders. Herein we report on the design, synthesis and biological evaluation of benzo[7]annulene- and [7]annulenothiophene-based selective and dual CCR2 and CCR5 receptor antagonists. Intermediates were designed in such a way that diversification could be introduced at the end of the synthesis. Starting from the lead compound TAK-779 (**1**), the quaternary ammonium moiety was exchanged by different non-charged moieties, the 4-methylphenyl moiety was extensively modified and the benzo[7]annulene core was replaced bioisosterically by the [7]annulenothiophene system. The naphthyl derivative **9h** represents the most promising dual antagonist (K_i (CCR2) = 25 nM, IC_{50} (CCR5) = 17 nM), whereas the 6-isopropoxy-3-pyridyl and 4-methoxycarbonylphenyl derivatives **9k** and **9r** show more than 20-fold selectivity for the CCR2 (K_i = 19 nM) over the CCR5 receptor.

Key words

CCR2 antagonists, CCR5 antagonists, dual antagonists, inflammation, TAK-779, late-stage diversification, bioisosterism, Suzuki-Miyaura cross-coupling, [^{3}H]TAK-779.

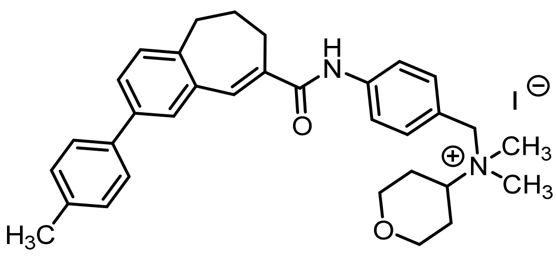
Introduction

The CCR2 and CCR5 chemokine receptor subtypes belong to the class A of G-protein-coupled receptors (GPCRs) of the CC chemokine type, which are characterized by high homology with rhodopsin.[1]

The CCR2 receptors exist in two alternatively spliced forms CCR2a and CCR2b, which differ in their C-terminal region that seems to determine their sensitivity towards G-proteins.[2] The CCR2 receptor binds the chemokines CCL2 (MCP-1) and CCL16 (MTN-1) as endogenous agonists. CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4) and CCL11 (eotaxin-1) are partial agonists[3-4] and CCL26 (MIP-4-α, eotaxin-3) acts as an antagonist on CCR2 receptors.[5] The CCR2 receptor is expressed on the surface of monocytes, basophils, dendritic cells, natural killer (NK) cells and activated T lymphocytes.[6] Data suggested that CCR2 plays a crucial role in the development of several diseases such as rheumatoid arthritis (RA),[7] inflammatory bowel disease,[8] atherosclerosis,[9] asthma,[10] diabetes type 2[11-12] and diabetic polyneuropathy.[13]

The CCR5 receptor binds the CC chemokines CCL2, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 and CCL26 as endogenous ligands.[14] CCL4 is a selective agonist on the CCR5 receptor,[15] while CCL26 and CCL7 act as endogenous antagonists.[5, 16] CCR5 receptors are expressed on the cell surface of a wide range of cell types including monocytes, macrophages, NK cells , dendritic cells and aortic smooth muscle cells.[17] In addition to its recognition of chemokines, the CCR5 receptor is acting as a co-receptor for HIV-1 entry into the target cells.[18] About 1 % of the Caucasian population is homozygous for a 32-base-pair deletion (Δ32) in the gene encoding the CCR5 receptor. This deletion causes a shift in the reading frame of the DNA triplets and creation of an early stop codon, which produces a truncated CCR5 receptor protein sticking in the endoplasmic reticulum. The naturally occurring CCR5Δ32 variant allows the analysis of the CCR5 knockdown effects in humans.[19-21] Homozygous individuals for CCR5Δ32 are remarkably resistant to HIV-1 infection. Heterozygotes for the CCR5Δ32 allele have lower density of CCR5 cell surface receptors and show delayed disease progression.[19] Furthermore, genetic epidemiology has shown a correlation between the lack of functional CCR5 receptors and the development of cardiovascular diseases.[22-25]

The CCR2 and CCR5 receptor share 71 % sequence identity, most of the differences are located in the extracellular and cytoplasmic loop regions.[26] CCR2 and CCR5 receptors are expressed on different cells, but in a complementary manner.[27-29] Both receptors play an important role in the trafficking of monocytes, macrophages and in the functions of other cell types relevant for the development and progression of several inflammatory, cardiovascular and autoimmune diseases.[7, 9-10, 22, 30] Strong preclinical evidence indicates greater efficacy for dual targeting of CCR2 and CCR5 receptors, than targeting either CCR2 or CCR5 alone.[9, 30-34]



1 (TAK-779)

Figure 1

Lead compound **1**.

Various potent CCR5 receptor ligands were reported to demonstrate high affinity to the CCR2 receptor as well and act as dual antagonists.[30, 35-36] Herein, benzo[7]annulene **1** (TAK-779, Figure 1), one of the first published small-molecule CCR5 antagonists with moderate CCR2 affinity[37] served as a starting point for the development of new, selective and dual CCR2 and CCR5 antagonists.

The poor bioavailability resulting from the quaternary ammonium ion was the major drawback in the development of **1** as a clinical candidate.[37-38]Therefore, we aimed to replace the quaternary ammonium moiety by different tertiary amines. Furthermore modifications of the benzo[7]annulene core structure and the 4-methylphenyl moiety were envisaged.

Results and discussion

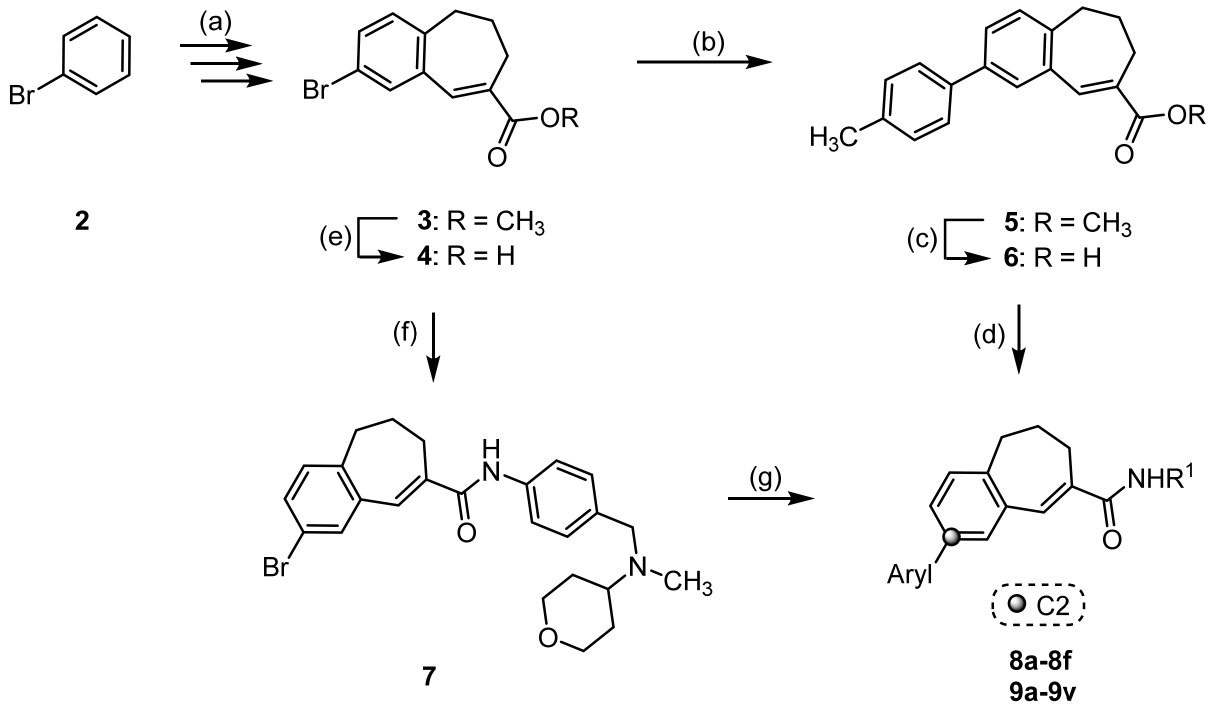
Synthesis

The brominated benzo[7]annulene-8-carboxylate **3** represents the key building block for the synthesis of benzo[7]annulene-based CCR5 antagonists **8a-8f** and **9a-v** (Scheme 1). It was obtained in a six-step procedure starting from bromobenzene (**2**).[39] In order to introduce structural diversity at the end of the synthesis ester **3** was further processed following two strategies.

According to the first strategy the 4-methylphenyl moiety of **1** was introduced by a Suzuki-Miyaura cross-coupling of **3** and 4-methylphenylboronic acid using $PdCl_2$(dppf) as catalyst, providing biaryl **5**. After hydrolysis of the ester **5**, amides **8a-8f** were prepared by COMU or HATU coupling of the resulting acid **6** with various primary amines.

In the second strategy, amide **7** was generated first by saponification of ester **3** with NaOH and subsequent COMU coupling of acid **4** with *N*-(4-aminobenzyl)-*N*-methyltetrahydro-2*H*-pyran-4-amine. Aryl bromide **7** was used as the central building block, since it allows diverse modifications in the last step of the synthesis via Suzuki-Miyaura coupling with various arylboronic acids. In order to obtain high yields of **9a-v** in the Suzuki-Miyaura cross-coupling reaction of **7**, the catalyst $PdCl_2$(dppf) was used and the base ($NaOCH_3$, K_2CO_3, KOAc) was optimized.[39]

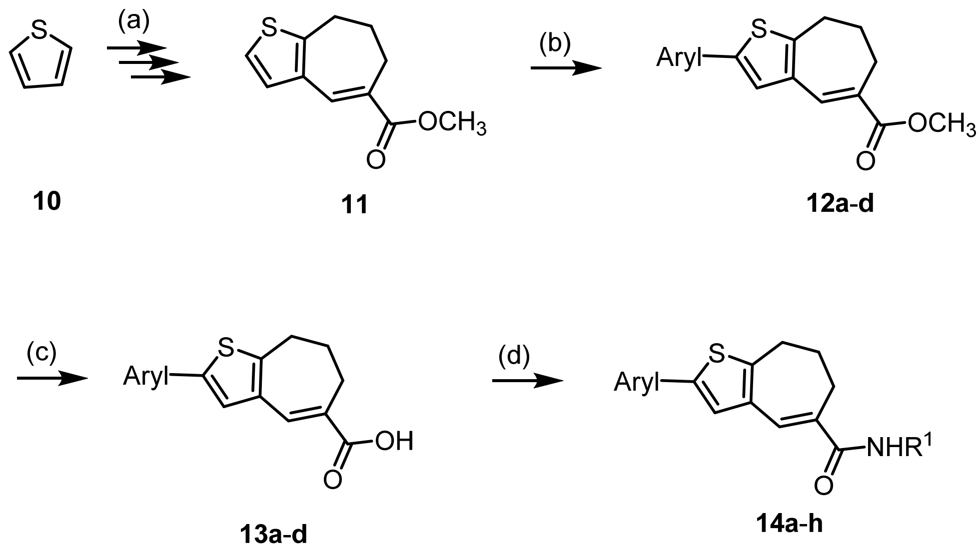
Scheme 1: Synthesis of benzo[7]annulen-8-carboxamides **8a-8f** with various amide N-substituents and **9a-v** with different aryl moieties at the 2-position.



Reagents and reaction conditions: (a) See ref.39. (b) 4-methylphenylboronic acid (1.1 eq.), KOAc (2 eq.), 5 mol% $PdCl_2$(dppf), DME, 100 °C, 12 h, 99 %. (c) 5 M NaOH, MeOH, reflux, 3 h, 97 %. (d) R^1NH_2, Et_3N (3 eq.), COMU (1.1 eq.) or HATU (1.1 eq.), CH_3CN or THF, rt, 12 h, 53 - 86 %; **8d** 25 %. (e) 5 M NaOH, MeOH, reflux, 3 h, 94 %.[39] (f) *N*-(4-aminobenzyl)-N-methyltetrahydro-2*H*-pyran-4-amine (1 eq.), Et_3N (3 eq.), COMU (1.1 eq.), CH_3CN, rt, 12 h, 77 %.[39] (g) base: $NaOCH_3$ (2 eq), K_2CO_3 (2 eq), or KOAc (2 eq), 5 mol% $PdCl_2$(dppf), DME, 100 °C, 12 h, 51 – 96 %. Definition of Aryl and R^1 is given in Tables 1 and 2.

Thiophene bioisosteres **14** of benzo[7]annulenes **8** and **9** were synthesized in a similar manner. The [7]annulenothiophene **11**, which was obtained in six reaction steps starting from thiophene (**10**),[40] served as the central building block (Scheme 2). In contrast to the bromobenzene derivatives **3** and **7** a direct C-H arylation at the α-position of the thiophene ring of **11** was envisaged. For this purpose the cationic iridium(I) complex $[Ir(cod)(py)PCy_3]PF_6$ (cod = 1,5-cyclooctadiene, py = pyridine),[41] known as the Crabtree catalyst[42,43] was used, which allowed direct introduction of various aryl moieties in the α-position *via* reaction of **11** with the corresponding aryl iodides.[40] The resulting esters **12a-d**, were hydrolyzed with NaOH and the acids **13a-d** were subsequently coupled with various amines to afford amides **14a-h**. Following this synthetic route, diversity was introduced during the direct C-H bond arylation of **11** and amide coupling of acids **13a-d**. Reversing of this synthetic route, *i.e.* arylation as the last reaction step, was not possible, since the direct C-H bond arylation failed using polyfunctional [7]annulenothiophenecarboxamides.[40]

Scheme 2: Synthesis of [7]annulenothiophenes **14a-h** with various aryl moieties and amide substituents.



12,13	Aryl
a	4-CH_3-Ph
b	3-CH_3-Ph
c	4-^{n}Bu-Ph
d	4-^{t}Bu-Ph

Reagents and reaction conditions: (a) see ref. 40. (b) iodobenzene derivative (1.4 eq.), $[Ir(cod)(py)PCy_3]PF_6$ (5 mol%), Ag_2CO_3 (1.1 eq.), 1,4-dioxane, 170 °C, 12 h.[40] (c) 5 M NaOH, MeOH, reflux, 3 h.[40] (d) R^1NH_2, HATU (1.1 eq.), Et_3N (2.0 eq.), THF or CH_3CN, rt, overnight, 64 – 88 %. Definition of Aryl and R^1 is given in Table 3.

Pharmacological activity

CCR2 Receptor affinity and antagonistic activity

Assays

In order to determine the binding affinity toward CCR2 receptors, all compounds were tested in radioligand displacement assays using membranes of U2OS cells stably expressing the human CCR2 receptor (U2OS-CCR2) and the iodinated endogenous agonist [^{125}I]-CCL2 as radioligand.[44] In addition to the binding affinity, the antagonistic activity of the ligands at the CCR2 receptor was determined in two complementary functional assays. For the human CCR2 receptor, an intracellular Ca^{2+}-flux assay with a CCR2B transfected Chem-1 cell line and recombinant human CCL2 was performed. In the β-arrestin recruitment assay, U2OS β-arrestin cell line transfected with murine CCR2 receptor and recombinant murine CCL2 were used. Of note, mouse CCR2 and human CCR2 receptors share 80 % sequence identity.[45] This limited conservation of the CCR2 receptor system across species might explain that in general the potencies of the compounds tested in the β-arrestin assay (mCCR2) are lower than in the Ca^{2+}-flux assay (hCCR2).

Structure affinity and structure activity relationships

Table 1

The receptor affinities of benzo[7]annulenes **1** and **8a**-**f** with different substituents at the amide N-atom are summarized in Table 1. The lead compound **1** displays an affinity of 2.0 nM in [^{125}I]-CCL2 radioligand binding assay and high potency in both the Ca^{2+}-flux (IC_{50} = 0.95 nM) and the β-arrestin recruitment assay (IC_{50} = 23 nM). The tertiary amine **8a**, derived from the quaternary ammonium compound **1** shows a remarkable CCR2 affinity (K_i = 18 nM) and high CCR2 inhibition in the Ca^{2+}-flux (IC_{50} = 1.9 nM) and β-arrestin assay

(IC_{50} = 33 nM). Since the tertiary amine **8a** was only 8-9-fold less potent than the quaternary ammonium compound **1**, it served as a new lead compound for further modifications.

In the first diversification round, the amino moiety of **8a** was replaced by different structurally related components (Table 1, compounds **8b-f**). Although various amines closely related to the amino group of **8a** were selected, receptor binding studies revealed that these variations are not tolerated by the CCR2 receptor.

Table 2

In the second diversification round the aryl moiety at 2-position of the benzo[7]annulene scaffold was varied extensively. (Table 2) Compound **9a**, bearing a phenyl ring without substituent, served as reference compound for the investigation of the influence of different aryl substituents on the CCR2 receptor affinity. It shows a considerable CCR2 affinity of 25 nM and high antagonistic activity in the Ca^{2+}-flux (IC_{50} = 3.1 nM) and β-arrestin assay (IC_{50} = 231 nM). Systematic analysis of the best position of the CH_3 group (**8a**, **9b**, **9c**) revealed **8a** with the methyl group in *p*-position being best recognized by the CCR2 receptor. Regioisomers **9b** and **9c** with the methyl group in *m*- and *o*-position display lower CCR2 affinity, but 2-fold higher potency in the Ca^{2+} mobilization assay (**9b**, IC_{50} = 0.73 nM; **9c**, IC_{50} = 0.8 nM). The bioisosteric replacement of the 4-methyphenyl moiety of **8a** by the 5-methylthienyl moiety (**9u**) led to a 2-fold lower CCR2 affinity (K_i = 43 nM).

Increasing the size of the alkyl group from methyl to *tert*-butyl group (**9f**) did not increase CCR2 receptor affinity. The impact of the size of the hydrophobic aryl substituent on the CCR2 receptor affinity was further investigated by the replacement of the *tert*-butylphenyl group (**9f**) by a biphenylyl moiety (**9g**), which led to an almost complete loss of CCR2 affinity. On the other hand, introduction of the 2-naphthyl group resulted in the highly potent CCR2 receptor ligand **9h** with a binding affinity of 25 nM and high potency of 1.7 nM in the Ca^{2+} mobilization assay and 24 nM in the β-arrestin assay.

The 3-pyridyl moiety (**9i**) and the 2-fluoro-3-pyridyl moiety (**9j**) were not tolerated by the CCR2 receptor.[39] However, introduction of the lipophilic isopropoxy residue into the 6-position of the pyridine ring resulted in the highly potent CCR2 antagonist **9k** with high affinity (K_i = 19 nM), high inhibition of Ca^{2+}-mobilization (IC_{50} = 1.7 nM) and β-arrestin recruitment (IC_{50} = 24 nM).

The introduction of a fluoro substituent at the *p*-position (**9l**) led to a reduction of the CCR2 affinity, which could not be improved by an additional methyl group adjacent to the *p*-fluoro substituent (**9m**). Rather polar substituents such as an OH-moiety (**9n,9o**), a hydroxymethyl moiety (**9q**), a formyl group (**9p**, **9v**) an amido group (**9s**) resulted in loss of CCR2 affinity and a significant decrease in CCR2 antagonistic activity.

Whereas **9r** with a methoxycarbonyl moiety instead of the *p*-methyl group of **8a** showed high CCR2 affinity (IC_{50} = 19 nM), it could not antagonize the CCR2 receptor in the Ca^{2+} mobilization and β-arrestin recruitment assay. In contrast, the weakly basic dimethylamine **9t** revealed the same CCR2 affinity (K_i = 17 nM) as the ester **9r** (K_i = 19 nM), but could also inhibit the Ca^{2+} flux and the β-arrestin recruitment. Altogether, the dimethylamine **9r** represents one of the most potent CCR2 ligands of this series of compounds.

Table 3

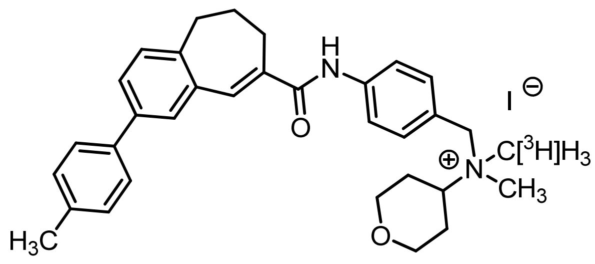
In Table 3 the affinity data of thiophene bioisosteres **14a-h** are summarized. The bioisosteric replacement of the benzo[7]annulene core with a [7]annuleno[b]thiophene system was not tolerated by the CCR2 receptor. Comparison of the CCR2 affinities of compounds **8a** (K_i = 18 nM) and **14a** (28 % inhibition of [^{125}I]-CCL2 binding at a concentration of 1 µM of **14a**) demonstrates a dramatic loss in CCR2 affinity after bioisosteric replacement of the benzene ring of **8a** by the thiophene ring of **14a**. The reduced CCR2 affinity might be attributed to the altered orientation of the 4-methylphenyl moiety at the thiophene annulated ring system due to the different bond angles of the five-membered thiophene ring. However, this effect could be compensated partly by different positioning of the methyl group at the phenyl moiety or by introduction of bulky substituents. The 3-methylphenyl derivative **14d** and the *tert*-butyl derivative **14h** reveal K_i-values of 109 nM and 270 nM, respectively.

CCR5 Receptor affinity

*Synthesis of the radioligand [^{3}H]**1***

Various assays to determine the interaction of ligands with CCR5 receptors are reported in the literature. Similar to the CCR2 binding assay, [^{125}I]-labeled CCL5 (RANTES) is often used as radioligand.[37,46] The disadvantages of these iodinated proteins as radioligands are their fast degradation by proteases and the rather short physical half-life of iodine-125 of 60 days. Therefore, we planned to use tritium-labeled **1** as radioligand,[47] since **1** has a high affinity towards CCR5 receptors (IC_{50} = 8.8 nM,[39] K_D ([^{3}H]TAK-779) = 30.2 ± 7.6 nM,[47] IC_{50} ([^{125}I]-CCL5) = 1.4 nM[37]), is stable in the presence of proteases and tritium has a long half-life of 12.3 years (Figure 2).

Figure 2: Tritium-labeled **1** ([^{3}H]**1**) used as radioligand in competitive binding assays.



[^{3}H]**1**

Tritium-labeled [^{3}H]**1** was prepared by methylation of tertiary amine **8a** with [^{3}H]H_3CI leading to a radiochemical purity of 99.8 % and a specific radioactivity of 2.9 GBq/µmol (79.1 Ci/mmol, custom synthesis performed by Perkin-Elmer). Herein, the CCR5 affinity of the test compounds was determined in competitive radioligand receptor binding assays. [^{3}H]-labeled **1** was used as radioligand and membrane fragments containing the CCR5 receptor as receptor material.

Structure affinity relationships

Replacement of the quaternary ammonium moiety of **1** (IC_{50} = 8.8 nM) by the corresponding tertiary amine (**8a**) led to 7-fold reduced but still high CCR5 affinity of 67 nM. (Table 1) Moderate CCR5 affinity was found for the piperidine derivative **8f** (IC_{50} = 269 nM), whereas all other modifications of the amide moiety resulted in complete loss of CCR5 affinity. The tertiary amine of **8f** occupies the same position in the CCR5 binding pocket as the quaternary ammonium moiety of **1**, demonstrating the importance of the position of the tertiary amine for high CCR5 affinity.

In Table 2 the CCR5 affinity of benzo[7]annulene derivatives with the standard amide moiety but different aryl moieties is summarized. The phenyl derivative **9a** without further substituents at the aryl moiety showed a moderate CCR5 affinity of 96 nM. The following structure affinity relationships of the compounds bearing various aryl moieties at 2-position of the benzo[7]annulene scaffold were detected: Whereas introduction of a *p*-methyl group at the phenyl moiety (**8a**: IC_{50} = 67 nM) seems to be beneficial for CCR5 interaction, regioisomers **9b** and **9c** with the methyl group in *m*- and *o*-position display slightly reduced CCR5 affinity. The bioisosteric replacement of the 4-methylphenyl moiety of **8a** by the 5-methylthienyl moiety in **9u** led to reduced CCR5 affinity. Larger alkyl substituents in the *p*-position of the phenyl moiety increased the CCR5 receptor affinity. The IC_{50}-values of the ethyl and *tert*-butyl derivatives **9d** and **9f** are 40 nM and 41 nM, respectively. Moreover, introduction of the 2-naphthyl group resulted in the highly potent CCR5 receptor ligand **9h** (IC_{50} = 17 nM).

Replacement of the methyl group at the *p*-position by a fluoro substituent (**9l**) retained the CCR5 affinity at the level of the methyl derivative **8a**. Addition of a methyl group adjacent to the *p*-fluoro substituent led to the very potent CCR5 receptor ligand **9m** (IC_{50} = 27 nM). A pyridyl ring instead of the phenyl ring (**9i-k**) or the introduction of very polar substituents (e.g. OH, CH_2OH, CH=O) appears to have detrimental effects on interactions with the CCR5 receptor. However, the dimethylamine **9t** (IC_{50} = 138 nM) is almost as potent as the methyl derivatives **8a** and **9b**.

The CCR5 affinity data in Table 3 indicate that the bioisosteric replacement of the benzo[7]annulene core with a [7]annuleno[*b*]thiophene system was not tolerated by the CCR5 receptor. Comparison of the CCR5 affinity of **8a** (IC_{50} = 67 nM) and **14a** (0 % inhibition of [^{3}H]TAK-779 binding at 1 μM) demonstrates the detrimental effect of the thiophene ring on CCR5 affinity.

CCR2/CCR5 Receptor selectivity

CCR2 and CCR5 receptors share 82 % sequence identity in their active sites.[48] The residues differing in the binding pockets of CCR2 and CCR5 receptors were analyzed to be

Ser101/Tyr89, His121/Phe109, and Arg206/Ile198.[48] Addressing the differences in the hydrophilic and electronic properties of the active site residues might be helpful in designing selective and dual CCR2 and CCR5 antagonists.

Replacement of the quaternary ammonium moiety of **1** with different tertiary amines reveals a possibility to obtain ligands with high CCR5 selectivity. Piperidine derivative **8f**, presumably occupying the same position in the CCR5 binding pocket as the quaternary ammonium moiety of **1**, was the only compound of this series with a slight preference for the CCR5 receptor. It shows moderate CCR5 receptor affinity (IC_{50} = 269 nM) but almost no CCR2 affinity (14 % inhibition of [^{125}I]-CCL2 at 1 μM).

Introduction of bulky alkyl substituents at the *p*-position of the phenyl ring does not significantly affect the CCR2/CCR5 receptor selectivity. However, introduction of a lipophilic isopropoxy residue into the 6-position of the pyridine ring led to the highly potent CCR2 ligand **9k** (K_i = 19 nM, IC_{50} (Ca^{2+}-flux) = 1.7 nM, IC_{50} (β-arrestin) = 24 nM) with only moderate CCR5 affinity (IC_{50} = 468 nM). The isopropoxy residue of **9k** is obviously able to interact with different residues of the active sites of CCR2 and CCR5 receptors.

Replacement of the methyl group at the *p*-position by a fluoro substituent (**9l**: IC_{50} = 115 nM) retained the CCR5 affinity at the level of the phenyl derivative **9a** (IC_{50} = 96 nM), but reduced the CCR2 affinity by 2-fold (**9a**: K_i = 25 nM, **9l**: K_i = 64 nM). Moreover, addition of a methyl group adjacent to the *p*-fluoro substituent led to the very potent CCR5 receptor ligand **9m** (IC_{50} = 27 nM) with reduced CCR2 affinity and potency (K_i = 90 nM, IC_{50} (Ca^{2+} influx) = 43 nM, IC_{50} (β-arrestin) = 560 nM). In contrast, introduction of an ester moiety resulted in a 4-fold loss in CCR5 affinity of **9r** compared to the phenyl derivative **9a**, but did not affect the CCR2 affinity.

Another strategy leading to improved CCR2 receptor selectivity involved the bioisosteric replacement of the benzo[7]annulene core with a [7]annuleno[b]thiophene system bearing a 3-methylphenyl group (**14d**, K_i = 109 nM) or a bulky *tert*-butyl group (**14h**, K_i = 270 nM). Both compounds displayed almost no CCR5 receptor affinity. The CCR2 receptor appears to be more tolerant for the different orientation of the aryl moieties at the five-membered thiophene ring.

σ Receptor affinity

The σ receptors show an unusually promiscuous ability to bind a variety of drugs with a central basic amino moiety flanked by at least two hydrophobic regions.[59-61] According to our experience with σ ligands, the novel compounds could fit into the σ pharmacophore. High affinity of the developed CCR2 and CCR5 receptor antagonists to the σ receptors could indicate potentially undesirable side effects in further development as drug candidates. Therefore the σ_1 and σ_2 affinity was recorded to get an idea about the off-rarget effects of these novel chemokine receptor ligands.

The σ_1 assay was performed with the radioligand [^{3}H]-(+)-pentazocine and membrane preparations obtained from guinea pig brains. For the σ_2 assay rat liver was used as receptor source and [^{3}H]di-o-tolylguanidine served as radioligand in the presence of an excess of (+)-pentazocine masking of σ_1 receptors.[62,63]

The σ_1 and σ_2 affinity data of all test compounds are summarized in Tables 1-3. Compounds with high CCR2 and CCR5 receptor affinity *e.g.* 4-methylphenyl derivative **8a**, 4-ethylphenyl derivative **9d** and 2-naphthyl derivative **9h** generally showed low σ_1 and σ_2 receptor affinities.

The 2-fluoro-3-pyridyl derivative **9j** showed the highest σ_1 receptor affinity (K_i = 28 nM) within this series of compounds with high selectivity (20-fold) over the σ_2 subtype (K_i = 566 nM), and a very low affinity for CCR2 and CCR5 receptors.

Conclusion

In order to develop novel TAK-779-derived, selective and dual-targeting CCR2 and CCR5 receptor antagonists, replacement of the quaternary ammonium moiety by non-charged analogous moieties, bioisosteric modification of the benzo[7]annulene core structure and extensive variations of the 4-methylphenyl moiety were performed.

Replacement of the quaternary ammonium moiety of **1** by the corresponding tertiary amine (**8a**) led to an approximately 7-9-fold decrease in both CCR2 and CCR5 binding affinity. Further amine modifications were not tolerated by both receptor subtypes. However, the reduced CCR2 and CCR5 affinity of **8a** was partially compensated by the introduction of large lipophilic aryl substituents at the 2-position such as a *p*-ethylphenyl (**9d**) or a 2-naphthyl moiety (**9h**). Thus **9h** represents the most promising dual CCR2 and CCR5 antagonist of this series of ligands with affinities of 25 nM (CCR2) and 17 nM (CCR5). In particular these dual targeting chemokine receptor antagonists represent promising candidates for the treatment of inflammatory, cardiovascular and autoimmune diseases.

The bioisosteric replacement of the benzo[7]annulene core with a [7]annuleno[b]thiophene system led generally to very low CCR2 and CCR5 affinity. However, the *m*-methylphenyl derivative **14d** and the *p*-*tert*-butylphenyl derivative **14h** show moderate CCR2 affinity without CCR5 affinity indicating remarkable selectivity for the CCR2 receptor over the CCR5 receptor. This observation is explained by a higher tolerance for the modified orientation of aryl moieties at the five-membered thiophene ring by the CCR2 receptor.

High CCR2 receptor affinity and selectivity was achieved by introduction of an isopropoxy residue in 6-position of the pyridine ring (**9k**) or a *p*-(methoxycarbonyl)phenyl residue (**9r**). Thus **9k** and **9r** represent promising selective CCR2 receptor antagonists.

Experimental

Chemistry general

Flash column chromatography (fc): Silica gel 60, 40–64 µm; parentheses include: diameter of the column, length of column, fraction size, eluent, R_f value. Melting point: melting point apparatus Stuart Scientific® SMP 3, uncorrected. ^{1}H NMR (400 MHz): Unity Mercury Plus 400 spectrometer (Varian®), AV400 (Bruker®), JEOL JNM-ECA-400; δ in ppm relative to tetramethylsilane; coupling constants are given with 0.5 Hz resolution, the assignments of ^{13}C and ^{1}H NMR signals were supported by 2D NMR techniques; MS: APCI = atmospheric pressure chemical ionization, EI = electron impact, ESI = electro-spray ionization: MicroTof (Bruker Daltronics, Bremen), calibration with sodium formate clusters before measurement. According to HPLC analysis the purity of all test compounds was greater than 95 %. HPLC method see Supporting Information.

General procedure A: Suzuki-Miyaura cross-coupling

A 20 mL Schlenk flask was equipped with a Dimroth condenser, a magnetic stirring bar and closed. The flask was flame-dried *in vacuo* and filled with N_2. Under a permanent flow of N_2, amide **7** (1 eq.), $PdCl_2$(dppf) (5 mol%), base (K_2CO_3, KOAc, $NaOCH_3$) (2 eq.) and arylboronic acid (1.1-1.5 eq.) were suspended in dry dimethoxyethane (5-15 mL). The flask was sealed and heated to reflux for 12 h. After cooling to rt, the mixture was filtered through a short silica pad (EtOAc). The filtrate was concentrated *in vacuo* to give the crude product, which was purified by fc. Recrystallization from acetonitrile afforded the final product.

N-[4-Diethylamino)phenyl]-2-(4-methylphenyl)-6,7-dihydro-5H-benzo[7]annulene-8-carboxamide (8b)

N^1,N^1-Diethylbenzene-1,4-diamine (60 mg, 0.36 mmol, 1 eq.) was added to a vigorously stirred mixture of acid **6** (100 mg, 0.36 mmol), triethylamine (73 mg, 0.72 mmol, 2 eq.) and HATU™ (153 mg, 0.40 mmol. 1.1 eq.) in THF (5 mL). The mixture was stirred overnight at rt. The mixture was concentrated *in vacuo* and the residue was purified by fc (EtOAc : CH_2Cl_2 = 1:2 + 5% MeOH) and recrystallized from acetonitrile to give **8b** as a colorless solid. R_f = 0.91 (MeOH : CH_2Cl_2 = 5:95), mp 153-155 °C , yield 86 mg (56 %). $C_{29}H_{32}N_2O$ (424.6 g/mol). HRMS (EI): *m/z* = calcd. for $C_{29}H_{33}N_2O$ [MH^+] 425.2587, found 425.2608. ^1H NMR ($CDCl_3$): δ (ppm) = 1.15 (t, J = 7.2 Hz, 6H, $N(CH_2CH_3)_2$), 2.15 (quint, J = 6.3 Hz, 2H, 6-CH_2), 2.40 (s, 3H, CH_{3tolyl}), 2.71 (t, J = 6.6 Hz, 2H, 7-CH_2), 2.81-2.92 (m, 2H, 5-CH_2), 3.34 (q, J = 7.0 Hz, 4H, $N(CH_2CH_3)_2$), 6.68 (d, J = 9.0 Hz, 2H, 3-CH_{phenyl}, 5-$CH_{phenyl.}$), 7.22 (d, J = 7.8 Hz, 1H, 4-CH), 7.25 (d, J = 8.4 Hz, 2H, 3-CH_{tolyl}, 5-CH_{tolyl}), 7.38-7.44 (m, 4H, 3-CH, 9-CH, 2-CH_{phenyl}, 6-$CH_{phenyl.}$), 7.47-7.50 (m, 3H, 2-CH_{tolyl}, 6-CH_{tolyl}, 1-CH), 7.51 (s, 1H, NH).

2-(4-Methylphenyl)-N-{4-[4-(tetrahydro-2H-pyran-4-yl)piperazin-1-yl]phenyl}-6,7-dihydro-5H-benzo[7]annulene-8-carboxamide (8c)

4-[4-(Tetrahydro-2H-pyran-4-yl)piperazin-1-yl]aniline (78 mg, 0.36 mmol, 1 eq.) was added to a vigorously stirred mixture of acid **6** (100 mg, 0.36 mmol), triethylamine (110 mg, 1.08 mmol, 3 eq.) and COMU™ (232 mg, 0.54 mmol. 1.5 eq.) in acetonitrile (5 mL). The mixture was stirred overnight at rt, during which a precipitate was formed. The solid was filtered off, washed with acetonitrile and water, dried and recrystallized from acetonitrile to afford **8c** as a colorless solid. R_f = 0.34 (MeOH : CH_2Cl_2 = 5:95), mp 248-250 °C (dec.), yield 107 mg (53 %). $C_{34}H_{39}N_3O_2$ (521.7 g/mol). HRMS (APCI): m/z = calcd. for $C_{34}H_{40}N_3O_2$ [MH^+] 522.3115, found 522.3092. ^1H NMR ($CDCl_3$): δ (ppm) = 1.62 (qd, J = 12.0/4.3 Hz, 2H, 3-$CH_{2pyran-axial,}$ 5-

$CH_{2pyran-axial}$), 1.76-1.86 (m, 2H, 3-$CH_{2pyran-equat}$, 5-$CH_{2pyran-equat}$), 2.15 (quint, J = 6.3 Hz, 2H, 6-CH_2), 2.40 (s, 3H, CH_{3tolyl}), 2.48 (tt, J = 10.0/2.9 Hz, 1H, 4-H_{pyran}), 2.67-2.78 (m, 6H, 7-CH_2, 3-$CH_{2piperazin}$, 5- $CH_{2piperazin}$), 2.82-2.92 (m, 2H, 5-CH_2), 3.13-3.28 (m, 4H, 2-$CH_{2piperazin}$, 6-$CH_{2piperazin}$), 3.41 (td, J = 11.9/1.9 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 4.05 (dd, J = 11.5/3.9 Hz, 2H, $CH_{2equat.}$-O-$CH_{2equat.}$), 6.93 (d, J = 8.9 Hz, 2H, 3-CH_{phenyl}, 5-CH_{phenyl}), 7.18-7.25 (m, 3H, 4-C*H*, 3-CH_{tolyl}, 5-CH_{tolyl}), 7.40 (s, 1H, 9-C*H*), 7.43 (dd, J = 7.8/1.9 Hz, 1H, 3-C*H*), 7.45-7.52 (m, 5H, 1-C*H*, 2-CH_{phenyl}, 6-CH_{phenyl}, 2-CH_{tolyl}, 6-CH_{tolyl}), 7.53 (s, 1H, N*H*).

_N_-{4-[_N_-Methyl-_N_-(tetrahydro-2_H_-pyran-4-yl)aminomethyl]phenyl}-6,7-dihydro-5_H_-benzo[7]annulene-8-carboxamide (9a)

According to general procedure A amide **7** (83 mg, 0.17 mmol), $PdCl_2$(dppf) (16 mg, 0.02 mmol, 10 mol %), $NaOCH_3$ (20 mg, 0.35 mmol, 2 eq.) and phenylboronic acid (24 mg, 0.2 mmol, 1.1 eq.) were suspended in dry dimethoxyethane (5 mL). The crude product was purified by fc (EtOAc : MeOH = 95:5) and recrystallized from acetonitrile to give **9a** as a colorless solid. R_f = 0.17 (CH_2Cl_2 : MeOH= 95:5), mp 165-167 °C, yield 74 mg (90 %). $C_{31}H_{34}N_2O_2$ (466.6 g/mol). HRMS (APCI): m/z = calcd. for $C_{31}H_{35}N_2O_2$ [MH^+] 467.2693, found 467.2690. ^1H NMR ($CDCl_3$): δ (ppm) = 1.55-1.84 (m, 4H, 3-CH_{2pyran}, 5-CH_{2pyran}), 2.11-2.22 (m, 2H, 6-CH_2), 2.23 (s, 3H, N-CH_3), 2.58-2.69 (m, 1H, 4-H_{pyran}),2.72 (t, J = 6.6 Hz, 2H, 7-CH_2), 2.84-2.96 (m, 2H, 5-CH_2), 3.37 (td, J = 11.7/2.3 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 3.60 (s, 2H, Ph-CH_2-N), 4.04 (dd, J = 11.4/2.5 Hz, 2H, $CH_{2equat.}$-O-$CH_{2equat.}$), 7.25 (d, J = 7.7 Hz, 1H, 4-C*H*), 7.30-7.38 (m, 4H, 3-$CH_{N-phenyl}$, 5-$CH_{N-phenyl}$, 3-C*H*, 9-C*H*), 7.37-7.47 (m, 3H, 4-$CH_{N-phenyl}$, 3-CH_{phenyl}, 5-CH_{phenyl}), 7.46 (d, J = 2.1 Hz, 1H, 1-C*H*), 7.54 (m, 2H, 2-$CH_{N-phenyl}$, 6-$CH_{N-phenyl}$), 7.57-7.60 (m, 2H, 2-CH_{phenyl}, 6-CH_{phenyl}), 7.65 (s, 1H, N-*H*).

2-(4-_tert_-Butylphenyl)-_N_-{4-[_N_-methyl-_N_-(tetrahydro-2_H_-pyran-4-yl) aminomethyl]phenyl}-6,7-dihydro-5_H_-benzo[7]annulene-8-carboxamide (9f)

According to general procedure A amide **7** (100 mg, 0.21 mmol), $PdCl_2$(dppf) (8 mg, 0.01 mmol, 5 mol%), KOAc (42 mg, 0.42 mmol, 2 eq.) and 4-*tert*-butylphenylboronic acid (45 mg, 0.25 mmol, 1.2 eq.) were suspended in dry dimethoxyethane (8 mL). The crude product was purified by fc (EtOAc : MeOH = 95:5) and recrystallized from acetonitrile to give **9f** as a colorless solid. (R_f = 0.22, CH_2Cl_2 : MeOH= 95:5), mp 164-165 °C, yield 88 mg (79 %). $C_{35}H_{42}N_2O_2$ (522.7 g/mol). HRMS (APCI): m/z = calcd. for $C_{35}H_{43}N_2O_2$ [MH^+] 523.3319, found 523.3335. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.36 (s, 9H, 2-CH_{3butyl}, 3-CH_{3butyl}, 4-CH_{3butyl}), 1.62-1.84 (m, 4H, 3-CH_{2pyran}, 5-CH_{2pyran}), 2.17 (t, *J* = 5.9 Hz, 2H, 6-CH_2), 2.21 (s, 3H, N-CH_3), 2.51-2.69 (m, 1H, 4-CH_{pyran}), 2.72 (t, *J* = 6.6 Hz, 2H, 7-CH_2), 2.84-2.94 (m, 2H, 5-CH_2), 3.37 (td, *J* = 11.5/2.3 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 3.57 (s, 2H, Ph-CH_2-N), 4.04 (dd, *J* = 10.9/4.1 Hz, 2H, CH_{2equat}-O-CH_{2equat}), 7.23 (d, *J* = 7.8, 1H, 4-C*H*), 7.31 (d, *J* = 8.4 Hz, 2H, 3-CH_{phenyl}, 5-CH_{phenyl}), 7.41-7.44 (m, 2H, 9-C*H*, 3-C*H*), 7.47 (d, *J* = 8.4 Hz, 2H, 3-C$H_{butylphenyl}$, 5-C$H_{butylphenyl}$), 7.51-7.54 (m, 3H, 1-CH, 2-C$H_{butylphenyl.}$ 6-C$H_{butylphenyl}$), 7.56 (d, *J* = 8.5 Hz, 2H, 2-H_{phenyl}, 6-H_{phenyl}), 7.62 (s, 1H, N-*H*).

_N_-{4-[_N_-Methyl-_N_-(tetrahydro-2_H_-pyran-4-yl)aminomethyl]phenyl}-2-(naphtalen-2-yl)-6,7-dihydro-5_H_-benzo[7]annulene-8-carboxamide (9h)

According to general procedure A amide **7** (100 mg, 0.21 mmol), $PdCl_2$(dppf) (8 mg, 0.01 mmol, 5 mol%), KOAc (40 mg, 0.42 mmol, 2 eq.) and 2-naphthylboronic acid (40 mg, 0.23 mmol, 1.1 eq.) were suspended in dry dimethoxyethane (5 mL).). The crude product was purified by fc (EtOAc : MeOH = 95:5) and recrystallized from acetonitrile to give **9h** as a colorless solid. R_f = 0.19, CH_2Cl_2 : MeOH= 95:5), mp 172-174 °C, yield 84 mg (77 %). $C_{35}H_{36}N_2O_2$ (516.6 g/mol). HRMS (APCI): m/z = calcd. for $C_{35}H_{37}N_2O_2$ [MH^+] 517.2850, found 517.2880. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.63-1.88 (m, 4H, 3-CH_{2pyran}, 5-CH_{2pyran}), 2.15-2.20 (m, 2H, 6-CH_2), 2.21 (s, 3H, N-CH_3), 2.64 (tt, *J* = 11.1/4.1 Hz, 1H, 4-C$H_{pyran,}$), 2.75 (t, *J* = 6.6 Hz, 2H, 7-CH_2), 2.87-2.97 (m, 2H, 5-CH_2), 3.37 (td, *J* = 11.6/2.4 Hz, 2H, CH_{2axial}-O-

CH_{2axial}), 3.57 (s, 2H, Ph-C*H*$_2$-N), 4.04 (dd, *J* = 10.8/4.3 Hz, 2H, CH_{2equat}-O-CH_{2equat}), 7.28-7.37 (m, 3H, *4-CH,* 3-C*H*$_{phenyl}$, 5-C*H*$_{phenyl}$), 7.45-7.54 (m, 3H, 9-C*H*, 6,7-C*H*$_{naphthyl}$), 7.55-7.61 (m, 3H, 3-CH, 2-C*H*$_{phenyl}$, 6-C*H*$_{phenyl}$), 7.63 (s, 1H, N-*H*), 7.68 (d, *J* = 2.0 Hz, 1H, 1-CH,), 7.74 (dd, *J* = 8.5/1.9 Hz, 1H, 3-C*H*$_{naphtyl}$), 7.82-7.98 (m, 3H, 1-C*H*$_{naphthyl}$, 5,8-C*H*$_{naphthyl}$), 8.04 (d, *J* = 1.8 Hz, 1H, 4-C*H*$_{naphthyl}$).

2-(6-Isopropoxypyridin-3-yl)-*N*-{4-[*N*-methyl-*N*-(tetrahydro-2*H*-pyran-4-yl)aminomethyl]phenyl}-6,7-dihydro-5*H*-benzo[7]annulene-8-carboxamide (9k)

According to general procedure A amide **7** (172 mg, 0.37 mmol), $PdCl_2$(dppf) (15 mg, 0.02 mmol, 5 mol%), KOAc (73 mg, 0.74 mmol, 2 eq.) and 6-isopropoxypyridine-3-ylboronic acid (100 mg, 0.55 mmol, 1.5 eq.) were suspended in dry dimethoxyethane (10 mL). The crude product was purified by fc (CH_2Cl_2: EtOAc + 5 % MeOH = 2:1) and recrystallized from acetonitrile to give **9k** as a colorless solid. R_f = 0.06 (CH_2Cl_2: EtOAc + 5 % MeOH = 2:1), mp 173-175 °C, yield 132 mg (67 %). $C_{33}H_{39}N_3O_3$ (525.7 g/mol). HRMS (APCI): m/z = calcd. for $C_{33}H_{40}N_3O_3$ [MH^+] 526.3064, found 526.3077. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.38 (d, 6.2 Hz, 6H, CH(C*H*$_3$)$_2$), 1.52-1.87 (m, 4H, 3-C*H*$_{2pyran,}$ 5-C*H*$_{2pyran}$), 2.07-2.20 (m, 2H, 6-C*H*$_2$), 2.21 (s, 3H, N-C*H*$_3$), 2.58-2.69 (m, 1H, 4-C*H*$_{pyran}$), 2.72 (t, *J* = 6.6 Hz, 2H, 7-C*H*$_2$), 2.81-2.98 (m, 2H, 5-C*H*$_2$), 3.37 (td, *J* = 11.6/2.3 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 3.57 (s, 2H, Ph-C*H*$_2$-N), 4.04 (dd, *J* = 11.5/4.3 Hz, 2H, CH_{2equat}-O-CH_{2equat}), 5.34 (sept. *J* = 6.2 Hz, 1H, C*H*(CH_3)$_2$), 6.75 (d, *J* = 8.6 Hz, 1H, 5-$CH_{pyridine}$), 7.24 (d, *J* = 8.1 Hz, 1H, 4-C*H*), 7.31 (d, *J* = 8.2 Hz, 2H, 3-C*H*$_{phenyl}$, 5-C*H*$_{phenyl}$), 7.37 (dd, *J* = 7.9/1.8 Hz, 1H, 3-C*H*), 7.40 (s, 1H, 9-C*H*), 7.44 (s, 1H, 1-C*H*), 7.56 (d, *J* = 8.4 Hz, 2H, 2-*H*$_{phenyl}$, 6-*H*$_{phenyl}$), 7.64 (s, 1H, N-*H*), 7.75 (dd, *J* = 8.6/2.6 Hz, 1H, 4-$CH_{pyridine}$), 8.35 (d, *J* = 2.6 Hz, 1H, 2-$CH_{pyridine}$).

2-(4-Methylphenyl)-*N*-{4-[*N*-methyl-*N*-(tetrahydro-2*H*-pyran-4-yl)aminomethyl]phenyl}-7,8-dihydro-6*H*-[7]annuleno[*b*]thiophene-5-carboxamide (14a)

N-(4-Aminophenyl)-*N*-methyltetrahydro-2*H*-pyran-4-amin (78 mg, 0.35 mmol, 1 eq.) was added to a vigorously stirred mixture of acid **13a**[40] (100 mg, 0.35 mmol), triethylamine (71 mg, 0.70 mmol, 2 eq.) and HATU™ (150 mg, 0.38 mmol. 1.1 eq.) in THF (5 mL). The mixture was stirred overnight at rt. The mixture was concentrated *in vacuo* and the residue was purified by fc (EtOAc : CH_2Cl_2 = 1:2 + 5% MeOH) and recrystallized from acetonitrile to give **14a** as a yellow solid. R_f = 0.13 (MeOH : CH_2Cl_2 = 5:95), mp 201 °C, yield 136 mg (80 %). $C_{30}H_{34}N_2O_2S$ (486.6 g/mol). HRMS (APCI): m/z = calcd. for $C_{30}H_{35}N_2O_2S$ [MH^+] 487.2414, found 487.2381. ^1H NMR ($CDCl_3$): δ (ppm) = 1.55-1.83 (m, 4H, 3-CH_{2pyran}, 5-CH_{2pyran}), 2.13 (quint, *J* = 5.1 Hz, 2H, 7-CH_2), 2.21 (s, 3H, N-CH_3), 2.36 (s, 3H, CH_{3tolyl}), 2.64 (tt, *J* = 10.9/3.5 Hz, 1H, 4-H_{pyran}), 2.84 (t, *J* = 5.8 Hz, 2H, 6-CH_2), 3.11 (t, *J* = 5.6 Hz, 2H, 8-CH_2), 3.37 (td, *J* = 11.6/2.3 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 3.57 (s, 2H, Ph-CH_2-N), 4.04 (dd, *J* = 11.4/4.4 Hz, 2H, CH_{2equat}-O-CH_{2equat}), 7.07 (s, 1H, 3-C*H*), 7.14-7.24 (m, 3H, 3-CH_{tolyl}, 5-CH_{tolyl}, 4-C*H*), 7.30 (d, *J* = 8.2 Hz, 2H, 3-CH_{phenyl}, 5-CH_{phenyl}), 7.42 (d, *J* = 8.1 Hz, 2H, 2-CH_{tolyl}, 6-CH_{tolyl}), 7.49-7.57 (m, 3H, 2-CH_{phenyl}, 6-CH_{phenyl}, N*H*).

2-(4-Methylphenyl)-*N*-[2-(tetrahydro-2*H*-pyran-4-yl)-1,2,3,4-tetrahydroisoquinolin-7-yl]-7,8-dihydro-6*H*-[7]annuleno[*b*]thiophene-5-carboxamide (14b)

2-(Tetrahydro-2*H*-pyran-4-yl)-1,2,3,4-tetrahydroisoquinolin-7-amine (82 mg, 0.35 mmol, 1 eq.) was added to a vigorously stirred mixture of acid **13a**[40] (100 mg, 0.35 mmol), triethylamine (71 mg, 0.70 mmol, 2 eq.) and HATU™ (150 mg, 0.38 mmol. 1.1 eq.) in THF (5 mL). The mixture was stirred overnight at rt. The mixture was concentrated *in vacuo* and the residue was purified by fc (EtOAc : CH_2Cl_2 = 1:2 + 5 % MeOH)) and recrystallized from acetonitrile to give **14b** as a yellow solid. R_f = 0.34 (MeOH : CH_2Cl_2 = 5:95), mp 215°C (dec.), yield 120 mg (69 %). $C_{31}H_{34}N_2O_2S$ (498.6 g/mol). HRMS (APCI): m/z = calcd. for $C_{31}H_{35}N_2O_2S$ [MH^+] 499.2414, found 499.2389. ^1H NMR ($CDCl_3$): δ (ppm) = 1.69 (dq, *J* = 12.1/4.2 Hz, 2H, 3-C$H_{2pyran-equat}$, 5-C$H_{2pyran-equat}$), 1.81-1.90 (m, 2H, 3-C$H_{2pyran-axial}$, 5-C$H_{2pyran-}$

$_{axial}$), 2.11 (quint???, J = 5.9 Hz, 2H, 7-CH_2), 2.36 (s, 3H, CH_{3tolyl}), 2.65 (tt, J = 11.1/3.8 Hz, 1H, 4-H_{pyran}), 2.77-2.90 (m, 6H, 6-CH_2, 3-CH_{2isoqu}, 4-CH_{2isoqu}), 3.09 (t, J = 5.3 Hz, 2H, 8-CH_2), 3.42 (t, J = 12.1 Hz, 2H, CH_{2axial}-O-CH_{2axial}), 3.77 (s, 2H, 1-CH_{2isoqu}), 4.06 (dd, J = 11.8/4.0 Hz, 2H, CH_{2equat}-O-CH_{2equat}), 7.05-7.08 (m, 2H, 3-CH, 5-CH_{isoqu}), 7.14-7.24 (m, 3H, 3-CH_{tolyl}, 5-CH_{tolyl}, 4-CH), 7.21 (dd, J = 8.2/2.2 Hz, 1H, 6-CH_{isoqu}), 7.41 (d, J = 7.7 Hz, 2H, 2-CH_{tolyl}, 6-CH_{tolyl}), 7.43 (d, J = 2.2 Hz, 1H, 8-CH_{isoqu}), 7.55 (s, 1H, NH).

CCR2 Assays

[^{125}I]-CCL2 Binding assays

Materials

[^{125}I]-CCL2 (81.4 GBq/µmol (2200 Ci/mmol)) was purchased from Perkin-Elmer (Waltham, MA). INCB3344 was synthesized as described previously.[49] [50] Tango CCR2-*bla* U2OS cells stably expressing human CCR2 were obtained from Invitrogen (Carlsbad, CA).

Cell culture and membrane preparation

U2OS cells stably expressing the human CCR2 receptor (Invitrogen, Carlsbad, CA) were cultured in McCoys5a medium supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mM non-essential amino acids (NEAAs), 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL G418, 50 µg/mL hygromycin, and 125 µg/mL zeocin in a humidified atmosphere at 37 °C and 5% CO_2. Cell culture and membrane preparation were performed as described previously.[44]

[^{125}I]-CCL2 Binding assays

Binding assays were performed in a 100-µl reaction volume containing 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS) and 15 µg of membrane protein at 37 °C. Nonspecific binding was determined with 10 µM INCB3344. Displacement assays were performed with 0.1 nM [^{125}I]-CCL2 using at least 6 concentrations of competing ligand for 150 minutes of incubation. The HP D300 digital dispenser from Tecan (Männedorf, Switzerland) was used to dispense the compounds in DMSO directly into the assay plate. Incubations were terminated by dilution with ice-cold 50 mM Tris-HCl buffer supplemented with 0.05% CHAPS and 0.5 M NaCl. Separation of bound from free radioligand was performed by rapid filtration through a 96-well GF/B filter plate precoated with 0.25% polyethylenimine using a PerkinElmer Filtermate-harvester (PerkinElmer, Groningen, The Netherlands). Filters were washed 10 times with ice-cold wash buffer, and 25 µl of Microscint scintillation cocktail (PerkinElmer) was added to each well; the filter-bound radioactivity was determined by scintillation spectrometry using the P-E 1450 Microbeta Wallac Trilux scintillation counter (PerkinElmer).

Data analysis

All experiments were analyzed using the nonlinear regression curve fitting program Prism 5 (GraphPad, San Diego, CA). For radioligand displacement data, K_i values were calculated from IC_{50} values using the Cheng and Prusoff equation.[51]

Functional CCR2 assays

Materials

Chem-1 cell line transfected with human CCR2 (ChemiSCREEN™ CCR2B Calcium-Optimized FLIPR Cell Line, Merck Millipore) was used for the intracellular calcium flux assay. U2OS β-arrestin cell line transfected with murine CCR2 (93-0543C3, DiscoveRx

Corporation, Ltd.) was used for the β-arrestin recruitment assay. Chemicals and reagents were purchased from different commercial sources and of analytical grade.

Measurement of intracellular calcium flux (G_q signaling pathway)

Chem-1 cells transfected with human CCR2 were cultured in DMEM high glucose medium (supplemented by 10 % FCS, 1 mM pyruvate, 15 mM HEPES, 500 µg/ml geniticine and non-essential amino acids (NEAA)). The cells were transferred into Optimem (supplemented by 5% FCS, 50 U/ml penicillin and 50 µg/ml streptomycin and NEAA) and seeded into 384-well plates (µCLEAR/black Greiner Bio One) at a density of 5000 cells/25 µl. Cells were incubated for approximately 24 h at 37 °C, 5 % CO_2. Before the assay medium was removed and the cells were incubated with Fluo-4 solution (25 µl Tyrode´s solution containing 3 µM Fluo-4 AM (1 mM DMSO stock solution), 0.4 mg/ml brilliant black, 2.5 mM probenicide, 0.03 % pluronic F-127) for 60 min at 37 °C, 5% CO_2. The compounds were dissolved in DMSO with 10 mM stock concentration followed by further dilution with DMSO in 1/3.16 steps. Required test solutions for the assay were obtained by dilution with Tyrode´s solution containing 2 mM $CaCl_2$ and 0,05 % BSA. Compounds (10 µL per well) were added and cells were incubated for 10 min at 37 °C, 5 % CO_2. Then 20 µl of agonist solution (recombinant human CCL2 (PeproTech, 300-04) in Tyrode´s solution with 0.05 % BSA) were added. CCL2 was applied at EC_{50}, which was determined in an experiment prior to compound testing (approximately 5 nM). Fluorescence intensity (excitation: 485 nm, emission: 520 nm) was measured for 120 s in 1.0 s intervals by a proprietary fluorescence measuring device. IC_{50} values were fitted using a 4 parameter logistic function (Hill function).

β-Arrestin recruitment assay

U2OS β-arrestin cell line transfected with murine CCR2 were cultured in MEM Eagle medium (supplemented by 10 % FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 250 µg/ml hygromycine and 500 µg/ml geniticine). The cells were transferred into Optimem (supplemented by 1 % FCS, 50 U/ml penicillin and 50 µg/ml streptomycin) and seeded into 384-well plates (µCLEAR/black Greiner Bio One) at a density of 2000 cells/25 µl. Cells were incubated for approximately 24 h at 37 °C, 5 % CO_2. The compounds were dissolved in DMSO with 10 mM stock concentration followed by further dilution with DMSO in 1/3.16 steps. Required test solutions for the assay were obtained by dilution with Tyrode´s solution containing 2 mM $CaCl_2$ and 0,05 % BSA. Compounds (10 µL per well) were added and cells were incubated for 10 min at 37 °C, 5 % CO_2. Then 20 µl of agonist solution (recombinant murine CCL2 (PeproTech, 250-10) in Tyrode´s solution with 0.05 % BSA) were added. CCL2 was applied at EC_{50}, which was determined in an experiment prior to compound testing (approximately 3 nM).

After 90 min of incubation at room temperature, 50 µL of detection reagent (93-001, DiscoveRx Corporation, Ltd.) per well were added. After additional 60 min of incubation at room temperature luminescent signal was detected by a proprietary luminescence-measuring device. IC_{50} values were fitted using a 4 parameter logistic function (Hill function).

CCR5 Radioligand receptor binding assay

Materials

The CCR5 receptor containing membrane homogenates were commercially available (MERCK Millipore, Darmstadt, Germany). Homogenizers: 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 Typ A and B. Scintillator: Meltilex (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany). Chemicals and reagents were purchased from different commercial sources and of analytical grade.

General protocol for the CCR5 binding assay

The assay was performed with the radioligand [^{3}H]TAK-779 (specific activity 2.9 GBq/µmol (79.1 Ci/mmol), custom synthesis by Perkin Elmer). The CCR5 receptor containing membrane fragments were used according to the instructions of the manufacturer and incubated with various concentrations of the test compound, 2 nM [^{3}H]TAK-779 and binding buffer (50 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.2 % BSA) at room temperature. The filtermats were washed with a buffer solution (50 mM HEPES pH = 7.4, 500 mM NaCl-solution and 0.1 % BSA). The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of the test compound in DMSO so that a 10 mM 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 h at room temperature before use. All binding experiments were carried out in duplicates 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 in various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L), 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 multiplates were shaken at a speed of 500-600 rpm at the specified temperature. The assays were terminated after 120 min by rapid filtration using the harvester.

During the filtration each well was washed five times with 300 μL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 min. After solidifying of the scintillator at room temperature, 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 IC_{50} values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis.

σ Receptor Assays

Details of the σ_1 and σ_2 assays are described in references[62,63].

Supporting Information

Purity data, general chemistry aspects, synthetic procedures, materials and experimental details for the assays, ^{1}H, ^{13}C and gHSQC NMR spectra, HPLC analysis, and MS spectra of all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Abbreviations

CCR2, CC chemokine receptor subtype 2; CCL2, CC chemokine ligand 2; CCR5, CC chemokine receptor subtype 5; COMU, (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate; HATU, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluoro-phosphate; TLC, thin layer chromatography.

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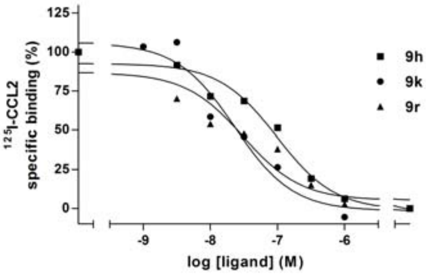
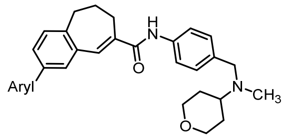
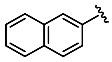
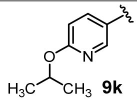
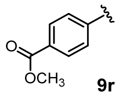
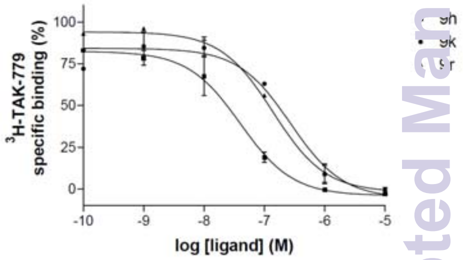
Table of Contents Entry



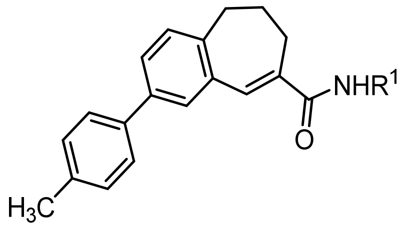


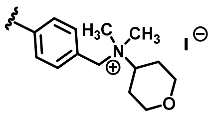
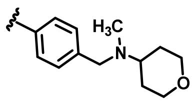
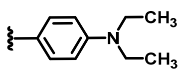
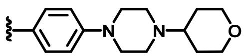
Aryl:	 9h	 9k	 9r
CCR2 IC$_{50}$ [nM]	25	19	19
CCR5 IC$_{50}$ [nM]	17	468	395

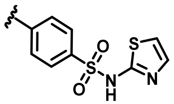
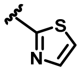
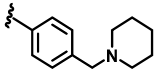


Late-stage diversification led to selective chemokine CCR2 receptor antagonists and dual-targeting CCR2/CCR5 receptor antagonists.

Table 1: Receptor affinities of benzo[7]annulenes **1** and **8a-8f** with different N-Substituents R^1.



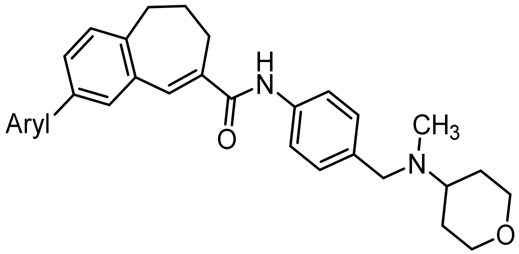
Compd.	R^1	CCR2			CCR5	σ_1	σ_2
		[^{125}I]-CCL2 displacement $K_i \pm$ SEM [nM] [a]	Ca^{2+} -flux (human CCR2) IC_{50} [nM][b]	β-arrestin recruitment (murine CCR2) IC_{50} [nM][b]	[^{3}H]TAK-779 displacement $IC_{50} \pm$ SEM [nM][c]	[^{3}H](+)-Pentazocine displacement K_i [nM]	[^{3}H]DTG displacement K_i [nM]
1[37]		2.0 ± 0.7	0.95	23	8.8 ± 1.7[d]	1730	1220
8a[39]		18 ± 5.5	1.9	33	67 ± 37	5 %	34 %
8b		0 %	263	5370	0 %	0 %	8 %
8c		0 %	920	19000	2 %	1070	1 %

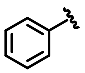
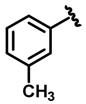
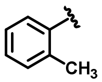
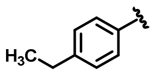
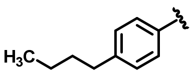
8d		1 %	52	907	15 %	0 %	0 %
8e		0 %	15000	30000	10 %	1 %	0 %
8f		14 %	517	5820	269	12 %	24 %

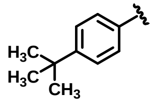
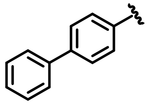
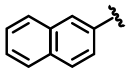
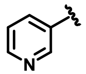
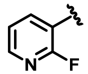
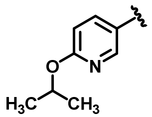
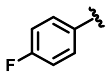
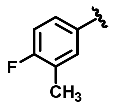
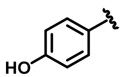
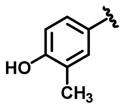
[a] K_i ± SEM (n = 3); [b] mean value of two experiments (n = 2); [c] IC_{50} ± SEM (n = 3); [d] four expermiments were performed (n = 4).

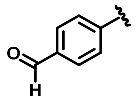
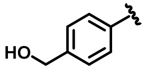
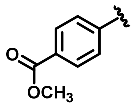
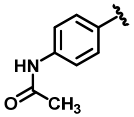
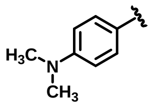
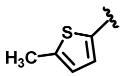
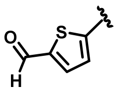
% values mean displacement (in %) of the radioligand binding at a concentraion of 1 μM of the test compound (n = 2).

Table 2: Receptor affinities of benzo[7]annulenes **9a-v** with different aryl moieties.



Compd.	Aryl	CCR2			CCR5	σ_1	σ_2
		[^{125}I]-CCL2 displacement K_i ± SEM [nM][a]	Ca^{2+} -flux (human CCR2) IC_{50} [nM][b]	β-arrestin recruitment (murine CCR2) IC_{50} [nM][b]	[^{3}H]TAK-779 displacement IC_{50} ± SEM [nM][c]	[^{3}H](+)-Pentazocine displacement K_i [nM]	[^{3}H]DTG displacement K_i [nM]
9a		25 ± 7.3	3.1	231	96 ± 27	661	500
9b		44 ± 9.5	0.73	67	106	231	18 %
9c		34 ± 4.9	0.80	195	139 ± 53	113	364
9d		26 ± 5.1	1.4	27	40 ± 19	1240	25 %
9e		34 ± 13	1.5	82	122	1060	0 %

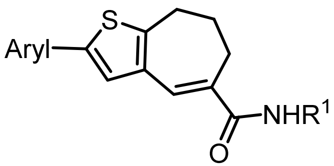
9f[39]		28 ± 12	0.9	119	41 ± 15	18 %	27 %
9g		45 %	156	3810	16000	39 %	5 %
9h		25 ± 6.6	1.7	24	17 ± 2	1680	799
9i[39]		43 %	93	2450	5900	189	22 %
9j		42 %	85	1480	37 %	28	566
9k		19 ± 4.2	2.7	90	468	177	541
9l		64 ± 11	5.4	203	115 ± 10	724	358
9m		90 ± 34	43	560	27 ± 7	224	362
9n[39]		35 %	82	1360	1500	155	252
9o		50 %	21	655	729	59	283

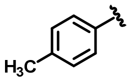
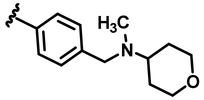
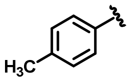
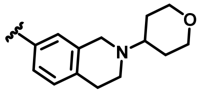
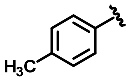
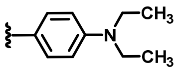
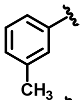
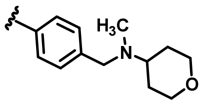
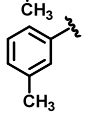
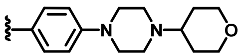
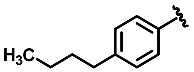
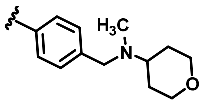
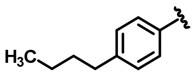
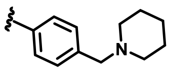
9p		37 %	125	2690	10 %	11 %	0 %
9q[39]		144 ± 18	35	11100	851	n.d.	n.d.
9r[39]		19 ± 9.4	12500	19200	395	238	2 %
9s[39]		25 %	815	4640	21 %	5 %	1240
9t[39]		17 ± 6.9	42	697	138	21 %	1840
9u[39]		43 ± 10	4.2	244	167 ± 66	661	500
9v		49 %	192	1970	1100	374	791

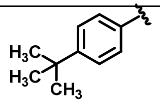
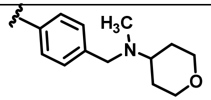
[a] K_i ± SEM (n = 3); [b] mean value of two experiments (n = 2); [c] IC_{50} ± SEM (n = 3).

% values mean displacement (in %) of the radioligand binding at a concentraion of 1 µM of the test compound (n = 2); n.d. = not determined.

Table 3: Receptor affinities of [7]annulenothiphenecarboxamides **14a-h**.



Compd.			CCR2			CCR5	σ_1	σ_2
			[^{125}I]-CCL2 displacement $K_i \pm$ SEM [nM][a]	Ca^{2+} -flux (human CCR2) IC_{50} [nM][b]	β-arrestin recruitment (murine CCR2) IC_{50} [nM][b]	[^{3}H]TAK-779 displacement $IC_{50} \pm$ SEM [nM][c]	[^{3}H](+)-Pentazocine displacement K_i [nM]	[^{3}H]DTG displacement K_i [nM]
14a			28 %	93	2510	0 %	385	14 %
14b			1 %	7930	30000	15 %	102	581
14c[40]			0 %	4.2	356	23 %	3 %	0 %
14d			109 ± 9.9	4.1	78	25 %	388	17 %
14e[40]			0 %	3.4	103	2 %	1550	0 %
14f[40]			31 %	5.4	2120	0 %	261	244
14g			0 %	n.d.	n.d.	10 %	3 %	8 %

14h			270 ± 20	4210	10400	32 %	195	10 %

[a] K_i ± SEM (n = 3); [b] mean value of two experiments (n = 2); [c] IC_{50} ± SEM (n = 3).

% values mean displacement (in %) of the radioligand binding at a concentraion of 1 μM of the test compound (n = 2); n.d. = not determined.