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CXCR3 antagonists: Quaternary ammonium salts equipped with biphenyl- and polycycloaliphatic-anchors

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ABSTRACT

Small-molecule ligands for the CXCR3 chemokine receptor receive considerable attention as a means to interrogate the roles of CXCR3 in vitro and in vivo and/or to potentially treat various conditions such as inflammatory diseases and cancer. We have synthesized and explored a novel class of small-molecule antagonists for CXCR3. A medium-throughput screen revealed an adamantane-guanidine as a hit. The guanidine unit was replaced by a small quaternary ammonium group, leading to ca. 5-fold increase in affinity. Substitution of the adamantane group by a myrtenyl moiety further increased affinity, while the benzyl group was decorated with an additional (substituted) aryl ring. This led to the identification of several bisaryl-based ligands with CXCR3 affinities of around 100 nM and with the ability to antagonize the functional activity of CXCL10.

1. Introduction

Chemokines are small endogenous peptides most notably involved in the recruitment of immune cells. They exert their action through activation of chemokine receptors, which belong to the superfamily of GPCRs. The group of reported chemokine receptors has steadily grown over the years and 19 members are currently known.[1,2] Blocking chemokine receptors by antagonists is thought to be associated with a large therapeutic potential, as a distorted immune system is involved in, for example, autoimmune diseases, inflammatory conditions and viral infections. A recent report reviews the development of chemokine receptor antagonists in more detail.[2]

CXCR3 is a prominent member of the class of chemokine receptors. CXCR3 is a 368-amino acid protein found on, for example, activated Th1 lymphocytes and on a small proportion of B cells and natural killer cells. The endogenous agonists for CXCR3 are the CXC chemokines CXCL9, CXCL10 and CXCL11, with the latter having the highest potency and efficacy. Based on expression levels and knockout studies, potential therapeutic roles for CXCR3 have been postulated in areas such as cancer and several autoimmune diseases.[3,4] A massive medicinal chemistry effort, mostly driven by the pharmaceutical industry, has revealed an increasing number of chemotypes as CXCR3 antagonists.[3–6] A selection of published antagonist structures is collected in Figure 1.[7–12] Reports on preclinical studies with such small-molecule antagonists (most notably **1**, AMG487) have disclosed positive results for some of the postulated roles of CXCR3 (e.g., in cancer and certain autoimmune diseases) whereas a controversy has emerged on the role of CXCR3 in organ transplant rejection.[4,13–15]

With the aim of obtaining tool compounds for our ongoing CXCR3 research,[11,16,17] we set out to develop and investigate a new CXCR3 antagonist scaffold. Toward this end, synthetic accessibility should be high and straightforward introduction of chemical diversity should be possible. In this paper, we report on such a scaffold class. Starting with a hit from a medium-throughput screening campaign, strategic changes in core structure were applied. This was followed by SAR studies which eventually led to the inception of a class of ammonium salts, all within excellent reach by the use of robust reactions.

2. Results and discussion

2.1. Screening campaign and design of a core structure

A proprietary library, containing 3360 pharmacologically active compounds, was screened at 10 μM for the ability to displace ^{125}I-CXCL10 from membranes of HEK293 cells stably

Abbreviations: IPAG, 1-(4-Iodophenyl)-3-(2-adamantyl)guanidine; DCE, 1,2-Dichloroethane; TEA, Triethylamine; hCXCR3, Human CXCR3; *cLogD*, Calculated Log *D*; GPCR, G protein-coupled receptor; TFA, Trifluoroacetic acid; HEK, Human embryonic kidney; SAR, Structure–activity relationship; rt, Room temperature.

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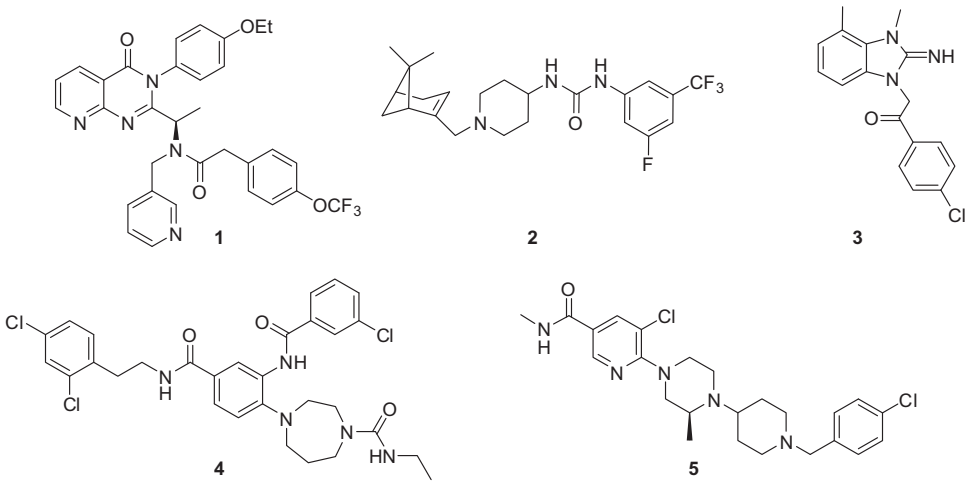


Figure 1. Structures of selected CXCR3 antagonist chemotypes.

expressing hCXCR3. The screening campaign had a hit-rate of 2.7%: 90 compounds out of 3360 gave >50% displacement at 10 μM. Figure 2 shows the results for a particularly rich plate. Compounds which inhibited specific binding of ^{125}I-CXCL10 for more than 50% were further evaluated by generation of full concentration-displacement curves.

Several interesting hits emerged from this medium-throughput screen. We were intrigued by 1-(4-iodophenyl)-3-(2-adamantyl)guanidine (IPAG, **6**), a sigma-receptor ligand (see Fig. 2).[18] IPAG gave a moderate CXCR3 affinity of pK_i = 5.4 in the ^{125}I-CXCL10 displacement assay. The structure of IPAG can be divided into three structurally different parts: (1) a highly lipophilic adamantane group, (2) a basic guanidine unit and (3) a 4-I-substituted phenyl group. The notion of the adamantane group coincided with our general interest for a potential CXCR3 pocket capable of accommodating polycycloaliphatic groups. Indeed, the finding of the adamantane being adjacent to a basic center in IPAG was integrated into a merging approach on which we have published, eventually giving rise to antagonists such as **7a**, **b**.[17] In the current study, we followed a conceptually different approach. The reported synthesis of compounds like IPAG involves reaction of the aniline hydrochloride with an adamantyl-cyanamide (by heating in the melt or refluxing for extended periods of time), the latter of which in turn has to be prepared by reaction with CNBr.[19] Such a synthetic approach is deemed non-optimal if chemical diversity is to be pursued. Hypothesizing that the basic guanidine unit may act (after protonation) as an important contributor to an ionic interaction with a CXCR3 residue, we decided to replace the guanidine with two different units capable of engaging in ionic interactions: a tertiary amine or a quaternary ammonium cation (Fig. 3). The distance between the aryl and adamantanyl units was kept similar to IPAG by inserting two CH_2 spacers. The resulting core scaffolds were used to introduce diverse chemical decoration, both at the polycycloaliphatic as well as at the aromatic anchor.

2.2. Synthesis

The syntheses of the tertiary amines and of the ammonium salts were highly integrated, with the former being the precursor for the latter. Thus, salts **8a–g**, **9a–j** and **10a–u** were all prepared in a single step from the corresponding tertiary amines **11a–g**, **12a–j** and **13a–u** (with **10p** being the sole exception, vide infra). The case of the exploration of the group is illustrative for this general synthetic approach (Scheme 1).

A reductive amination between any of the aldehydes **14–19** (syntheses shown in Supplementary data) and 4-iodobenzylamine hydrochloride using NaBH(OAc)$_3$ delivered secondary amine **20** (31–69%).[20] Subsequent Eschweiler–Clark methylation afforded the tertiary amine **11** (74%-quant.). Last, methylation of **11** with an excess of MeI in the dark followed by cold precipitation with DCM/*t*BuOMe afforded the iodide salt **8** (15–92%). Although conversions were always high in the MeI-reactions described here and in following paragraphs, isolated yields varied considerably due to, for example, varying solubilities of the product salts in

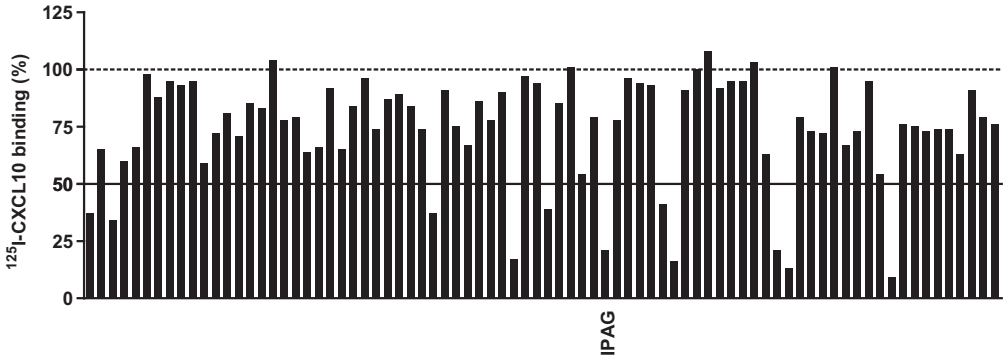


Figure 2. A part of the screening campaign, displaying one 96-well plate (80 compounds). Each bar represents a compound (10 μM) from the library. Data are presented as percentage specific ^{125}I-CXCL10 binding to hCXCR3. Non-specific binding was determined in the presence of the CXCR3 antagonist **1** (10 μM). Inhibition of ^{125}I-CXCL10 binding by IPAG is indicated.

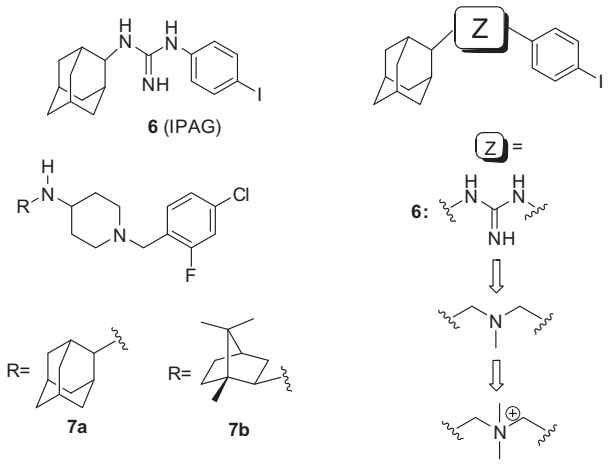


Figure 3. Design strategy based on the hit IPAG (**6**).

the precipitation medium. Introduction of one ethyl group was accomplished by reductive amination between **20f** and acetaldehyde to **11g**, followed by a similar methylation to salt **8g**. A second ethylation of **11g** with EtI failed under several conditions, most likely due to the steric crowding imposed upon the nitrogen atom.

The synthesis of the majority of compounds with varying aromatic side chains (Scheme 2) followed a strategy which is similar to the one depicted in Scheme 1. That is, myrtenal (**19**) was reductively aminated with various commercially available amines to **21a**, **c**, **e–j** (20–39%). Compounds **21a**, **c**, **e–j** were subsequently methylated under Eschweiler–Clark conditions to tertiary amines **12a**, **c**, **e–j** (43–97%). A more convenient and efficient approach for the syntheses of compounds like **12** was initiated at the stage of the 2-iodo-compound **12b**. That is, large-scale imine formation between **19** and H_2NMe followed by $NaBH_4$-based reduction afforded building block **22**, which was reductively alkylated by 2-iodobenzaldehyde to yield **12b**. Cyclic tertiary amine **12d** was prepared by reductive alkylation of tetrahydroisoquinoline with **19**. All syntheses were finalized by methylation of **12a–j** to salts **9a–j** as described (31–91%).

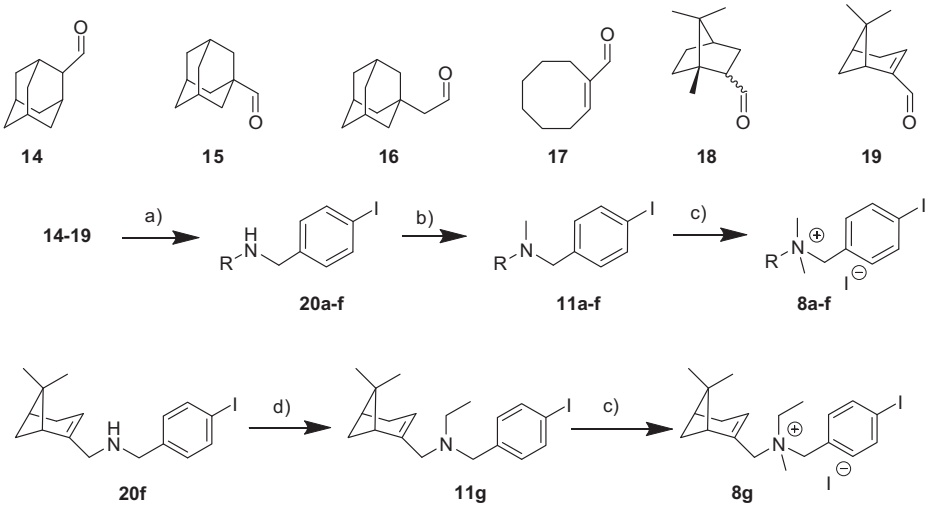
A Suzuki reaction was envisioned as the key reaction in the syntheses of functionalized bisaryls **13a**, **f–u** (Scheme 3). This approach required bromo-containing building blocks **12g** and **23**. Compound **23** was obtained by the sequence starting from **22** as discussed for **12b**. The same held true for the synthesis of **12g**, which was scaled up to 10 g without the need for chromatography using this alternative route. Gratifyingly, reaction of **12g** or **23** with many arylboronic acids under microwave Suzuki conditions smoothly afforded the tertiary bisaryl-amines **13a**, **f–o**, **q–u** and **24** (30–79%). No interference of the myrtenyl double bond was encountered in these reactions. Precursors **13b–e** were prepared from **22** and the corresponding commercially available aldehydes (39–90%). Once again, all syntheses were completed by reaction of **13a–o**, **q–u** with MeI to the salts **10a–o**, **q–u** (29–89%). For an aniline substituent, compatibility problems were foreseen and we opted to use Boc-protected anilines **24** and **25** as surrogates throughout the sequence. Target anilines **13p** and **10p** were obtained from **24** and **25** by standard TFA-mediated deprotection and by convenient acid-free microwave heating of an aqueous mixture,[21] respectively.

The large majority of ammonium salts comprised solids that were readily soluble in DMSO, MeCN or $CHCl_3$. The stability of selected salts in solution was evaluated and it was shown that these salts possess good stability in DMSO or MeOH/H_2O (see Supplementary data).

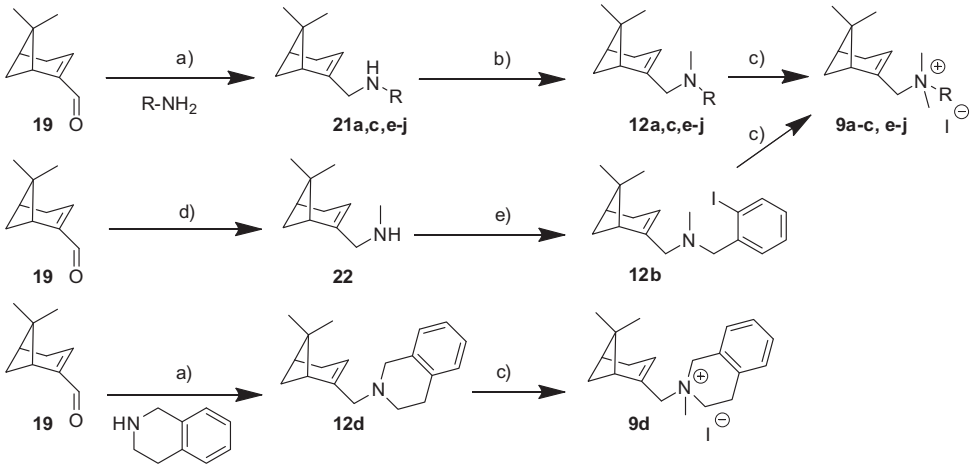
2.3. Binding affinities

All synthesized compounds were tested for their affinity for hCXCR3 by measuring the displacement of ^{125}I-CXCL10 binding to membranes of HEK293 cells stably expressing hCXCR3.[16] In this assay, the reference CXCR3 antagonist AMG487 (**1**) gives a pK_i value of 7.4 ± 0.1 (n = 19), while CXCL10 binds CXCR3 with a pK_d value of 9.7 ± 0.1 (n = 4).

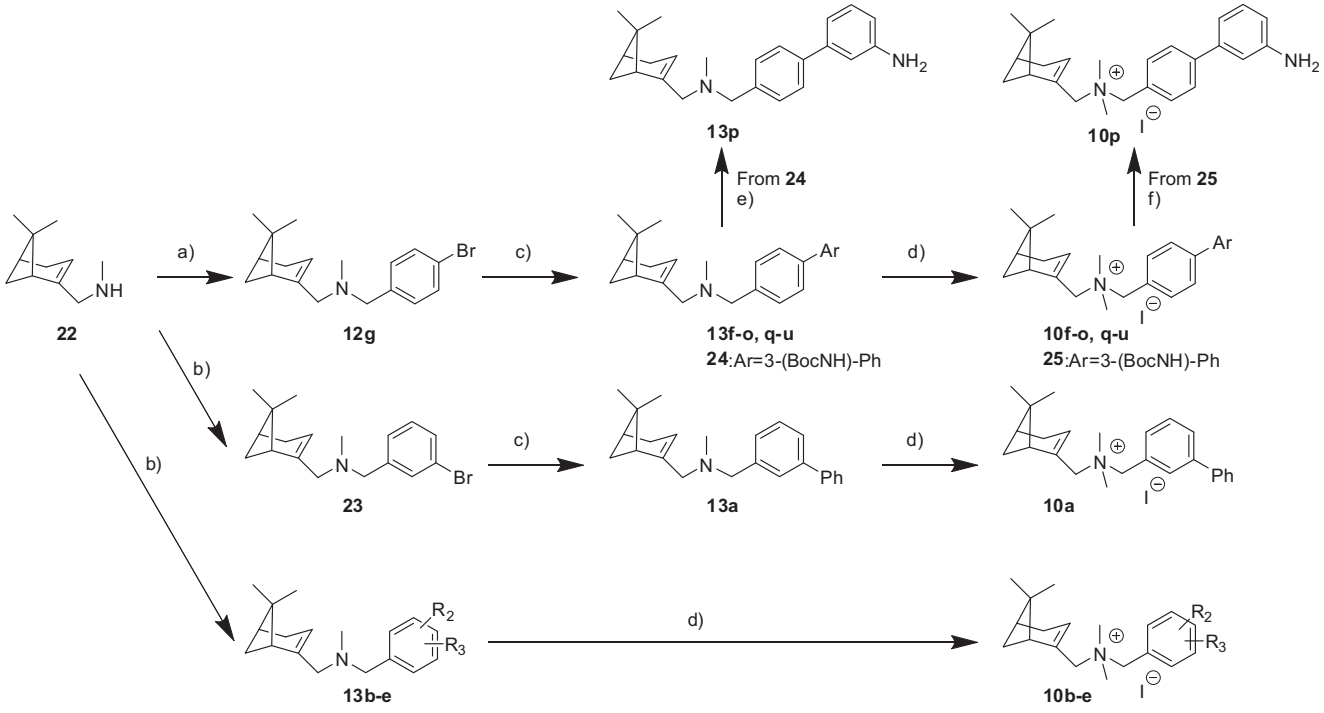
As shown in Figure 3, the design strategy involved replacement of the guanidine in IPAG by a tertiary amine and subsequently by a quaternary ammonium cation. Since the secondary amine was an intermediate en route to these two compounds, it was also included in our initial pharmacological analysis. As Table 1 shows, this secondary amine (**20a**) gave a slightly higher affinity compared to IPAG, but the tertiary amine (**11a**) led to 0.5 log unit drop in affinity. More interestingly, methylation to a permanent cation (**8a**) lifted the pK_i substantially above that of IPAG (from 5.4 to 6.1). While it is known that increasing lipophilicity, for example



Scheme 1. Synthesis of compounds with varying cycloaliphatic groups and of *N*-ethyl analogues. Reagents and conditions: (a) 4-iodobenzylamine hydrochloride, $NaBH(OAc)_3$, DCE, TEA, rt, 1–2 days; (b) HCOOH, 37% aq H_2CO, 2–4 h, reflux; (c) [1] DCM, MeI, 1–3 days, rt [2] DCM/*t*BuOMe, 0 °C, filtration; (d) MeCHO, $NaBH(OAc)_3$, DCE, 2.5 h, tr, 65%.



Scheme 2. Synthesis of compounds with varying aromatic groups. Reagents and conditions: (a) $NaBH(OAc)_3$, DCE, (TEA), rt, 1–2 d; (b) HCOOH, 37% aq. H_2CO, 2–4 h, reflux; (c) [1] DCM, MeI, 1–3 d, rt [2] DCM/*t*BuOMe, 0 °C, filtration. (d) [1] H_2NMe (33% in EtOH), MeOH, 1 d, rt [2] $NaBH_4$, 3 h, rt, 89%. (e) $NaBH(OAc)_3$, 2-iodobenzaldehyde, DCE, rt, 20 h, 36%.



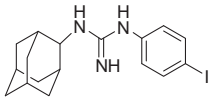
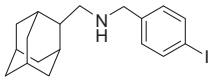
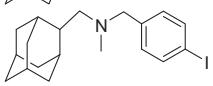
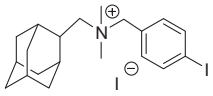
Scheme 3. Synthesis of compounds with varying bisaryl groups. Reagents and conditions: (a) [1] 4-BrPhCHO, $NaBH(OAc)_3$, DCE, rt, 20 h [2] HCl, Et_2O [3] recrystallisation, 71% as HCl salt; (b) substituted benzaldehyde, $NaBH(OAc)_3$, DCE, rt, 1–2 days; (c) $Pd(PPh_3)_4$, $ArB(OH)_2$, Na_2CO_3, *i*PrOH, toluene, H_2O, μW, 1.5 h; (d) [1] DCM, MeI, 1–3 days, rt [2] DCM/*t*BuOMe, 0 °C, filtration; (e) TFA, DCM, rt, 1 h, 88%; (f) H_2O, μW, 2 h, 88%.

by N-methylation, can increase the affinity of protein ligands, the calculated Log*D* values for homologues **20a**, **11a** and **8a** (Table 1) clearly suggest that lipophilicity is not the major contributor to their affinities. Studies with the tetra-alkyl-ammonium salt TAK-779 and the CCR5 chemokine receptor have shown that the nature of the binding of a tetra-alkyl-ammonium cation could involve a subtle mix of ionic interactions with a negatively charged residue and cation–π interactions with aromatic residues.[22] In analogy, we believe that the ability of our core to engage in (an) electrostatic interaction(s) plays a dominant role in the observed affinities. Indeed, sequential N-methylation has been used before as a probe for electrostatic interactions in other proteins.[23,24] Since all of our ammonium salts synthetically required the tertiary amine precursors, we were well suited to test a variety of such tertiary amines, the affinities of which will also be reported throughout the manuscript. This enabled the investigation of the general validity of our hypothesis dictating (an) electrostatic interaction(s) to be a key contributor to CXCR3 affinity.

Having established the quaternary ammonium cation as a suitable and synthetically accessible core, the stage was set for decoration of the peripheral groups. As mentioned, the notion of the adamantane in IPAG coincided with our search for a potential 'polycycloaliphatic' CXCR3 pocket[17] and this led us to first probe the 'left-hand' side (Table 2).

A shift of the adamantane toward the 1-position (**8b**) led to lower affinity than for **8a**, but it could be restored by chain elongation

Table 1
hCXCR3 affinities of key compounds involved in the design strategy based on the IPAG hit

Compound	Structure	*c*Log*D*[a]	pK_i ± SEM[b]
6 (IPAG)		1.98	5.4 ± 0.0
20a		2.22	5.6 ± 0.1
11a		2.85	5.1 ± 0.1
8a		1.02	6.1 ± 0.1

[a] Calculated for pH 7.4 using the standard protocol of ChemAxon.
[b] Measured by the displacement of ^{125}I-CXCL10 binding to membranes of HEK293 cells stably expressing hCXCR3 (n = 2–4, each in duplicate).

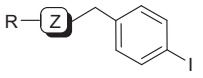
(**8c**). Introduction of a cyclooctenyl (**8d**) or a bornyl group (**8e**) had no advantage. However, a significant finding was the beneficial effect exerted by the myrtenyl group[8] in compound **8f** (pK_i = 6.6). Two considerations are of interest with respect to this finding. First, the advantageous effect of a myrtenyl group over a 2-adamantanylmethylene unit is opposite of what we observed with 4-amino-piperidines.[17] Second, while N-methylation adds 1.1 log unit to the tertiary base in our case (compare **11f** and **8f**), N-methylation on myrtenyl-urea **2** has been reported by others not to increase the potency.[8] Collectively, this evidence suggests that the polycycloaliphatic head of the current IPAG-derived set is targeting the receptor in a different manner compared to the 4-amino-piperidine and 1-aryl-3-piperidin-4-yl-urea series.

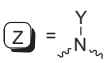
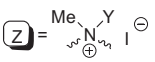
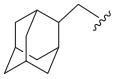
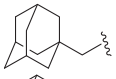
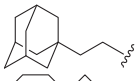
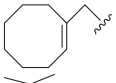
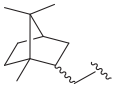
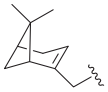
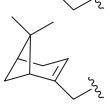
With the myrtenyl group having lifted the pK_i to attractive values, we decided to briefly return to the nitrogen center and investigate the effect of N-substituents larger than Me. We had already encountered steric crowding during our synthetic attempts toward N,N-diethylation (vide supra). In line with this observation, the steric bulk imposed upon the N-atom by a Me and Et group in **8g** may shield the cation from fully interacting with the protein resulting in a drop in pK_i (compare **8f** and **g**).

Attention was then devoted to the aromatic 'right-hand' side of **8f** (Table 3). An iodine-scan (**8f**, **9a–b**) established the *para*-position as the preferred anchor for substitution. The advantage of a suitable *para*-substituent was also evident from the inferior affinity of benzyl-analogue (**9c**). A constrainment exercise on **9c** by incorporation of a tetrahydroisoquinoline substructure (giving **9d**) provided no obvious surplus value and this approach was not pursued further. A *para*-halogen-scan (**8f**, **9e–g**) revealed that all halogens except F were tolerated. Upon further elaboration with the synthetically more accessible Cl-derivatives, we found that neither double Cl-substitution (**9h**) nor chain elongation (**9i**) improved affinity. The data in Table 3 suggests that there is still room for growth beyond a halogen at the *para*-position of the benzyl substituent. This inspired us to apply a strategic switch toward a biphenyl scaffold. The affinity of **9j** (pK_i = 6.5) revealed that this switch was allowed, opening the door for SAR efforts on the newly added phenyl ring (Table 4).

Just as with the case of iodides **8f** versus **9a**, the preference for a *para*-attachment over *meta*-attachment was reinforced within the biphenyl system (**9j** vs **10a**). The relative orientation (i.e., torsion angle) and interactions between the biphenyl rings may also play

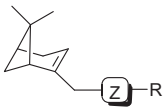
Table 2
hCXCR3 affinities of compounds with varying cycloaliphatic groups

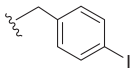
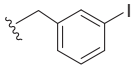
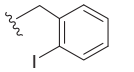
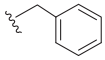
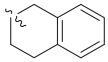
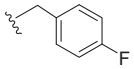
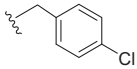
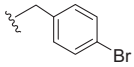
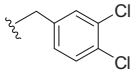
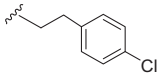
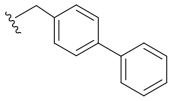


R	Y	Z = 	pK_i ± SEM[a]	Z = 	pK_i ± SEM[a]
	Me	**11a**	5.1 ± 0.1	**8a**	6.1 ± 0.1
	Me	**11b**	n.d.[b]	**8b**	5.7 ± 0.3[c]
	Me	**11c**	5.3 ± 0.2	**8c**	6.2 ± 0.1
	Me	**11d**	5.1 ± 0.2	**8d**	6.0 ± 0.1
	Me	**11e**[d]	5.1 ± 0.0	**8e**[d]	5.8 ± 0.2
	Me	**11f**	5.5 ± 0.1	**8f**	6.6 ± 0.1
	Et	**11g**	5.4 ± 0.1	**8g**	6.2 ± 0.1

[a] For assay conditions, see footnotes of Table 1.
[b] Affinity could not be accurately measured due to poor solubility resulting from high crystallinity.
[c] The relatively large SEM results from poor solubility due to high crystallinity.
[d] Mixture of four stereomers.

Table 3
hCXCR3 affinities of compounds with varying aromatic groups



R	Z = 	pK_i ± SEM[a]	Z = 	pK_i ± SEM[a]
	**11f**	5.5 ± 0.1	**8f**	6.6 ± 0.1
	**12a**	5.1 ± 0.2	**9a**	5.7 ± 0.2
	**12b**	5.3 ± 0.0	**9b**	5.8 ± 0.1
	**12c**	4.8 ± 0.0	**9c**	5.4 ± 0.1
	**12d**[b]	4.9 ± 0.2	**9d**[b]	5.6 ± 0.2
	**12e**	5.2 ± 0.1	**9e**	5.9 ± 0.2
	**12f**	5.6 ± 0.2	**9f**	6.7 ± 0.2
	**12g**	5.5 ± 0.1	**9g**	6.5 ± 0.1
	**12h**	5.5 ± 0.1	**9h**	6.4 ± 0.1
	**12i**	5.3 ± 0.1	**9i**	6.0 ± 0.1
	**12j**	5.5 ± 0.1	**9j**	6.5 ± 0.2

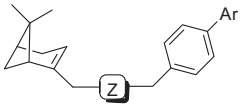
[a] For assay conditions, see footnotes of Table 1.
[b] No N-Me (**12d**) or only one N-Me (**9d**) substituent is present, as the N is part of a tetrahydroisoquinoline ring.

important roles. A few approaches were unleashed on **9j** to investigate this further. Insertion of an O-atom to reduce inter-ring interactions (**10b**) led to almost a log unit decrease in affinity. Constrainment of **9j** and **10b** (affording **10c**–**e**) did not surpass the affinity of **9j**, although the 0.6 log unit increase from **10b** to **e** is of note. Clearly, the unconstrained biphenyl system lent itself best to subsequent studies and the remainder of the efforts involved singly linked bisaryl systems.

An array of substituents with varying characteristics was installed on the 'outer' phenyl ring to elucidate the SAR around this moiety. A comparison between a 3-F and 2-F group (**10f** vs **g**) as well as between a 3-CF_3 and a 4-CF_3 group (**10h** vs **i**) pointed toward the *meta*-position as the preferred anchor. Nonetheless, *meta*-substitutions with carbonyl-containing groups (**10j**, **k**) were not of surplus value. The same held true for a selected *meta*, *para*-disubstituted compound (**10l**). The fact, however, that **10f** had an affinity similar to **9j** caused us to shift our attention toward a broader set of small *meta*-substituents (**10m–q**). Significant progress was made in this area. For example, the 3-Me- and 3-NO_2-derivatives (**10m**, **n**) afforded good affinities of pK_i = 6.4 and 6.7, respectively. On the other hand, protic polar substituents such as –OH and –NH_2 (**10o**, **p**) reduced the affinity, most notably in the case of the –NH_2 group. This could be (partially) the result of a higher enthalpic penalty for desolvation of **10o**, **p** from an aqueous environment. Indeed, a move towards chlorine substituents restored the affinity, with a *meta*-chloro pattern (**10q**) providing an excellent affinity (pK_i = 6.9). For **10q**, the *meta*-position was reinforced as the better anchor than the *para*-position (compare **10q**–**r**). Addition of an extra chloro-group to **10q** (i.e., **10s**) led to a drop in affinity. Last, two selected heteroaromatic rings were tried (**10t**, **u**), resulting in good affinities (pK_i = 6.6 and 6.7, respectively) for these heteroaromatic bisaryl compounds.

Collectively, the presented affinity evidence shows the advantageous effect of specific polycycloaliphatic groups on affinity for CXCR3.[17] The data also reveal that the unconstrained biphenyl-moiety is a versatile scaffold for SAR studies on CXCR3. A *meta*-group on the outer ring of this moiety is beneficial, with an inspection of the best compounds revealing that a non-protic, medium-sized *meta*-substituent works best. Compound **10q** emerges as the best ligand, but it is fair to say that the biphenyl moiety of the current class of ammonium salts generally exerts subtle CXCR3 effects on overall CXCR3 affinity, both from a steric and electronic point of view. A consistent picture that emerges is that of a clear increase in affinity upon methylation of the tertiary amine to a quaternary ammonium salt. This can be deduced from three different series: **11** → **8**, **12** → **9** and all measured examples

Table 4
hCXCR3 affinities of compounds with varying bisaryl groups



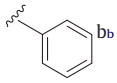
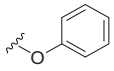
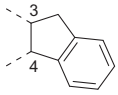
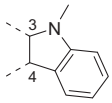
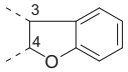
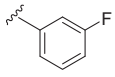
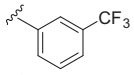
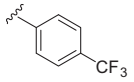
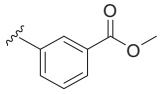
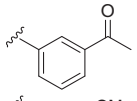
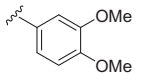
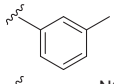
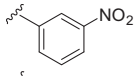
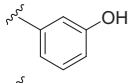
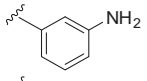
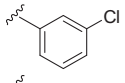
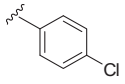
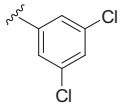
Ar	Z = NMe	pK_i ± SEM[a]	Z = N^+MeMe I$^-$	pK_i ± SEM[a]
	**12j**	5.5 ± 0.1	**9j**	6.5 ± 0.2
	**13a**[b]	n.d.[d]	**10a**[b]	5.5 ± 0.2
	**13b**	n.d.[d]	**10b**	5.7 ± 0.2
	**13c**[c]	n.d.[d]	**10c**[c]	6.3 ± 0.2
	**13d**[c]	n.d.[d]	**10d**[c]	6.0 ± 0.1
	**13e**[c]	n.d.[d]	**10e**[c]	6.3 ± 0.1
	**13f**	5.6 ± 0.1	**10f**	6.5 ± 0.1
	**13g**	n.d.[d]	**10g**	6.2 ± 0.0
	**13h**	n.d.[d]	**10h**	6.3 ± 0.0
	**13i**	n.d.[d]	**10i**	5.9 ± 0.2
	**13j**	n.d.[d]	**10j**	6.0 ± 0.2
	**13k**	n.d.[d]	**10k**	6.2 ± 0.1
	**13l**	n.d [d]	**10l**	6.3 ± 0.0
	**13m**	n.d.[d]	**10m**	6.4 ± 0.1
	**13n**	5.7 ± 0.1	**10n**	6.7 ± 0.1
	**13o**	n.d.[d]	**10o**	5.8 ± 0.2
	**13p**	5.2 ± 0.1[e]	**10p**	5.5 ± 0.1
	**13q**	5.4 ± 0.1	**10q**	6.9 ± 0.1
	**13r**	n.d.[d]	**10r**	6.3 ± 0.0

Table 4 (*continued*)

Ar	Z = NMe	pK_i ± SEM[a]	Z = NMe$_2^+$ I$^-$	pK_i ± SEM[a]
	**13s**	n.d.[d]	**10s**	6.4 ± 0.1
	**13t**	5.2 ± 0.1	**10t**	6.6 ± 0.1
	**13u**	5.4 ± 0.0	**10u**	6.7 ± 0.2

[a] For assay conditions, see footnotes of Table 1.
[b] Ar-group on *meta*-position.
[c] This is a tricyclic 'biphenyl'-system, with the enumerations 3 and 4 for the two linkages referring to their position with respect to the aromatic carbon which is connected to the NCH_2 carbon.
[d] Affinities for tertiary amine precursors, belonging to salts having a pK_i <6.5, were not determined.
[e] Although the corresponding salt **10p** had a pK_i <6.5, compound **13p** had been prepared as a final product and was therefore tested.

from **13** → **10**. Such an increase not uncommonly involves more than 0.9 log unit (see Fig. S1). This underscores the importance of a quaternary cationic center in interacting with the chemokine receptor[22] and also strongly suggests that all presented compounds bind CXCR3 by very similar means.

2.4. ^{125}I-CXCL11 displacement and functional activity

A subset of compounds was inspected for additional pharmacological characteristics on hCXCR3. The set consisted of two interesting members of each different subclass: 4-chlorophenyl salt **9f**, 4-iodophenyl salt **8f**, chlorobiphenyl salt **10q** and thienylphenyl salt **10u**.

Since CXCL11 is another endogenous ligand for CXCR3, the selected compounds were tested in an assay using ^{125}I-CXCL11 with standards being **1** (pK_i = 6.8 ± 0.2, n = 3) and CXCL11 (pK_d = 10.3 ± 0.0, n = 2). The results show that all four compounds were able to displace ^{125}I-CXCL11 from hCXCR3 (Table 5). The CXCR3-binding ability of initial hit IPAG (**6**) was reconfirmed in this assay as well (pK_i = 5.4, Table 5).

The activity (expressed as pK_b values) of the compounds in a functional assay was measured by their inhibitory effect on [^{3}H]-inositolphosphates levels upon stimulation of HEK293T-hCXCR3/Gα_{qi5} cells with 50 nM CXCL10, as reported by us before.[16] The results in Table 5 and Figure 4 clearly show that all four compounds, as well as reference **1**, were able to inhibit the activity of CXCL10 on CXCR3 in a concentration-dependent manner. Thus, these compounds can be classified as CXCR3 antagonists. Although slight variations exist, the pK_i and pK_b values generally fall within the same range.

Table 5
Additional pharmacological data on hCXCR3 of representative compounds

Compound	p$K_{i,\ CXCL10}$ ± SEM[a]	p$K_{i,\ CXCL11}$ ± SEM[b]	pK_b ± SEM[c]
6 (IPAG)	5.4 ± 0.0	5.4 ± 0.0	n.d.
8f	6.6 ± 0.1	6.4 ± 0.2	6.6 ± 0.2
9f	6.7 ± 0.2	6.3 ± 0.2	6.3 ± 0.1
10q	6.9 ± 0.1	6.5 ± 0.0	6.3 ± 0.2
10u	6.7 ± 0.2	6.5 ± 0.1	6.4 ± 0.1
1 (AMG487)	7.4 ± 0.1	6.8 ± 0.2	8.0 ± 0.0

[a] As shown in Tables 1–4.
[b] Measured by the displacement of ^{125}I-CXCL11 binding to membranes of HEK293 cells stably expressing hCXCR3 (n = 2–4, each in duplicate).
[c] Measured by the dose-dependent inhibitory effect on [^{3}H]-inositolphosphates levels after stimulation with 50 nM CXCL10 in HEK293T-hCXCR3/Gα_{qi5} cells (n = 2–4, each in duplicate). n.d. = not determined.

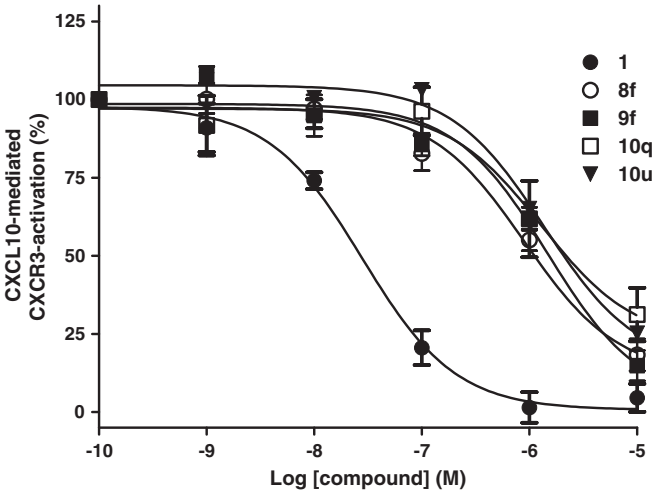


Figure 4. Grouped functional curves for antagonistic activities of representative compounds on hCXCR3. Footnote c of Table 5 contains assay details.

3. Conclusion

This paper describes SAR explorations based on an initial guanidine hit (IPAG) found by a medium-throughput screen on hCXCR3. Strategic replacement of the guanidine core by amine- and ammonium-moieties led to two postulated guanidine surrogates. Of these, a quaternary ammonium core clearly surpassed the affinity of IPAG and provided a new benzyl-ammonium CXCR3-scaffold. Versatile synthetic routes collectively provided access to ~50 members, allowing efficient SAR studies on many structural features of this scaffold (measured by inhibition of ^{125}I-CXCL10 binding). Most notably, installing a polycycloaliphatic myrtenyl group and expansion of the benzyl group to a (substituted) biphenyl unit led to subtle effects in binding and provided affinities up to ~100 nM (**10q**). Representative members inhibited ^{125}I-CXCL11 binding to CXCR3 and prevented activation of CXCR3 by CXCL10 in a functional assay.

The consistent increase in affinity upon methylation of the tertiary amine precursors to the quaternary ammonium salts suggests that electrostatic interactions contribute significantly to the

affinities of the ammonium salts. Furthermore, our results reinforce our previously disclosed notion that certain polycycloaliphatic-anchors are beneficial for CXCR3 affinity. The described biphenyl-scaffold may serve as a novel tool in CXCR3 research.

4. Experimental

4.1. General notes on synthetic procedures

4.1.1. Chemicals

Unless reported otherwise, all chemicals were from Aldrich. Hit compound IPAG (**6**) was obtained from Tocris Bioscience (HR-MS: calcd for M+H+($C_{17}H_{23}IN_3$): 396.0931, found: 396.0939). 9-Methyl-9*H*-carbazole-2-carbaldehyde was from Matrix. AMG487 (**1**) has been prepared as reported by us before.[25] THF, toluene and CH_2Cl_2 were distilled freshly from CaH_2, all other solvents were used as received.

4.1.2. Synthesis

Unless indicated otherwise, all reactions were carried out under an inert atmosphere. TLC analyses were performed with Merck F254 Alumina Silica Plates using UV visualization or staining. Column purifications were carried out manually using Silicycle Ultra Pure Silica Gel or automatically using the Biotage® equipment. Microwave reactions were carried out on a Biotage® Initiator. All HR-MS spectra were recorded on Bruker micrOTOF MS using ESI in positive ion mode. The ^1H-, ^{13}C- and 2D-NMR spectra were recorded on a Bruker 200, 250, 400 or 500 MHz spectrometer. Melting points were taken using the Standford Research Systems Optimelt apparatus and values given are uncorrected. Systematic names for molecules were generated using Chemdraw Version 11. Unless reported otherwise, all compounds have a purity $\geqslant$95% as measured by LC–MS. Analytical LC–MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV–vis detector with the MS detection performed with a Shimadzu LC–MS-2010 liquid chromatograph mass spectrometer. The column used is an Xbridge (C18) 5 μm column (100 mm × 4.6 mm). Compound purities were calculated as the percentage peak area of the analyzed compound by UV detection at 230 nm. The buffer mentioned is a 0.4% (w/v) NH_4HCO_3 solution in water, adjusted to pH 8.0 with NH_4OH.

4.1.3. Typical elution mode for quaternary ammonium salts

Solvent B (90% MeCN–10% buffer) and solvent A (90% water–10% buffer), flow rate of 2.0 mL/min, start 5% B, linear gradient to 90% B in 10 min, then 15 min at 90% B, then 10 min at 5% B, total run time of 35 min. The iodide counter ion is also visible by UV.

4.1.4. Typical elution mode for tertiary amines

Solvent B (90% MeCN–10% buffer) and solvent A (90% water–10% buffer), flow rate of 2.0 mL/min, isocratic mode at 10% solvent A and 90% solvent B, total run time of 30 min.

4.2. Procedure A: General procedure for reductive alkylation of 4-iodobenzylamine hydrochloride

The aldehyde (1 mmol) and 4-iodobenzylamine hydrochloride (0.270 g, 1 mmol) were mixed with dry DCE (25 mL). If indicated, TEA (139 μL, 1 mmol) was added to liberate the parent amine. Next, $Na(AcO)_3BH$ (0.318 g, 1.5 mmol) was added and the mixture was stirred at rt for 24–36 h. Aq NaOH-soln (2 M) was added and the mixture was stirred vigorously for 10 min, after which DCM was added. The organic layer was separated and the aq layer was back-extracted with DCM (1×). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. If required, the crude product was purified by column chromatography.

4.3. Procedure B: General procedure for Eschweiler–Clark N-methylation

The secondary amine (0.13 mmol) was heated at reflux for 2–4 h in a mixture of formic acid (2 mL) and 37% formaline (2 mL). The reaction mixture was allowed to cool to room temperature, and volatiles were removed by evaporation. The crude product was mixed with aq NaOH-solution (ca. 10 mL, 2.0 M), and extracted with DCM (3×). The organic layers were dried (Na_2SO_4), filtered and concentrated. If required, the product was purified by column chromatography.

4.4. Procedure C: General procedure for methylation to a quaternary ammonium salt

In a round bottom flask, the tertiary amine precursor (0.25 mmol) was dissolved in dry DCM (6.4 mL). The flask was placed under a nitrogen atmosphere, closed with a septum and covered with aluminum foil. Then, iodomethane (31 μL, 0.50 mmol) was added to the solution via a syringe. The reaction mixture was allowed to stir at room temperature overnight in the dark. TLC analysis was used to monitor the progress of the reaction. If required, additional quantities of iodomethane (1–2 equiv) were added followed by prolonged stirring. Such cycles were applied until completion of the reaction. Next, the reaction mixture was cooled in an ice bath and 3 volume equivalents of *t*BuOMe were added to the reaction mixture slowly via a dropping funnel while stirring. This usually caused precipitation of the salt. The precipitate was filtered and washed with a precooled solution of DCM/*t*BuOMe (1:3), after which the salt was dried. If required, the salt was recrystallised. This whole isolation procedure proved robust and delivers the product salt in high purity (although formation of solvates sometimes occurs). Isolated yields varied due to, for example, varying solubilities in DCM/*t*BuOMe (1:3). Occasionally, in the initial precipitation with *t*BuOMe the salt does not precipitate as a solid but as a sticky oil. In those cases, the solvent is decanted from the oil after *t*BuOMe addition is complete. The remaining oil was washed with a precooled solution of DCM/*t*BuOMe (1:3) by stirring, settling and decanting. All ammonium salts were stored in the dark.

4.5. Procedure D: General procedure for reductive amination of myrtenal

The amine (3.3 mmol) and myrtenal (19, 0.5 g, 3.3 mmol) were mixed with dry DCE (10 mL). If the amine is a salt, TEA (460 μL, 3.3 mmol) was added to liberate the parent amine. Next, $Na(AcO)_3BH$ (1.06 g, 5.00 mmol) was added and the mixture was stirred at rt for 48 h. Aq NaOH-soln (2 M) was added and the mixture was stirred vigorously for 10 min, after which DCM was added. The organic layer was separated and the aq layer was back-extracted with DCM (1×). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The crude product was purified by column chromatography.

4.6. Procedure E: General procedure for reductive alkylation of compound 22

Amine **22** (0.3 g, 1.8 mmol) and the aldehyde (1.0 mmol) were mixed with dry DCE (4 mL). Next, $Na(AcO)_3BH$ (0.53 g, 2.5 mmol) was added and the mixture was stirred at rt for 20 h. Aq NaOH-soln (1.0 M, ca. 10 mL) was added and the mixture was stirred vigorously for 10 min, after which DCM was added. The organic layer

was separated and the aq layer was back-extracted with DCM (1×). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. The crude product was purified by column chromatography.

4.7. Procedure F: General Suzuki procedure

A microwave vial was charged with the arylbromide (**12g** or **23**, 300 mg, 0.90 mmol), the boronic acid ($ArB(OH)_2$, 1.80 mmol), toluene (3 mL), *i*-PrOH (0.8 mL), and a 2.0 M Na_2CO_3-solution (0.50 mL). This mixture was degassed for 15 min by bubbling N_2 through the mixture using a needle. Next, $Pd(PPh_3)_4$ (52 mg, 0.045 mmol) was added to the mixture and the vial was immediately capped and again degassed for 5 min. The vial was heated in the microwave for 90 min at 130 °C. After this, the reaction mixture was filtered over Celite and most of the organic solvents were evaporated in vacuo. The pH of the residue was adjusted to pH >11 with a 1.0 M aq NaOH solution and the mixture was extracted with DCM (3×). The combined DCM layers were washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo. This afforded the crude product, which was purified by column chromatography.

4.8. Procedures for pharmacological assays

4.8.1. Cell lines

HEK293 cells stably expressing human CXCR3 were a kind gift from Dr. Erik Boddeke en Dr. Knut Biber (University Medical Center Groningen).

4.8.2. Library screen using ^{125}I-CXCL10

The binding experiment was performed as described before,[16] with the exception that membranes were prepared from HEK293 cells stably expressing human CXCR3.[26] 10 μM of test compound was used.

4.8.3. Affinity measurements on hCXCR3 using ^{125}I-CXCL10

pK_i values at hCXCR3 were determined as described before,[16] with the exception that membranes were prepared from HEK293 cells stably expressing human CXCR3.[26]

4.8.4. Affinity measurements on hCXCR3 using ^{125}I-CXCL11

pK_i values at hCXCR3 were determined as described before,[16] with the exception that membranes were prepared from HEK293 cells stably expressing human CXCR3.[26]

4.8.5. Functional activity through phospholipase C (PLC) activation

The pK_b values were determined as described before.[16]

4.9. Data analysis

Non-linear regression analysis of the data and calculation of pK_d and pK_i values was performed using Prism 4.03. The pK_b values in the PLC activation assay were calculated using the Cheng–Prusoff equation $pK_b = IC_{50}/(1 + [agonist]/EC_{50})$.

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Supplementary data

Supplementary data (figure for ΔpK_i between quaternary ammonium salts and tertiary amines; detailed syntheses and characterization of all compounds; stability data; copies of representative 1D/2D NMR spectra and chromatogram) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.04.035.

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