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# Ligand-Biased and Probe-Dependent Modulation of Chemokine Receptor CXCR3 Signaling by Negative Allosteric Modulators

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Over the last decade, functional selectivity (or ligand bias) has evolved from being a peculiar phenomenon to being recognized as an essential feature of synthetic ligands that target G protein-coupled receptors (GPCRs). The CXC chemokine receptor 3 (CXCR3) is an outstanding platform to study various aspects of biased signaling, because nature itself uses functional selectivity to manipulate receptor signaling. At the same time, CXCR3 is an attractive therapeutic target in the treatment of autoimmune diseases and cancer. Herein we report the discovery of an 8-azaquinazolinone derivative (*N*-{1-[3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]ethyl}-4-(4-fluorobutoxy)-*N*-[(1-methylpiperidin-4-yl)methyl]butanamide, **1 b**) that can inhibit CXC chemokine 11 (CXCL11)-dependent G protein activation over β-arrestin recruitment with 187-fold selectivity. This compound also demonstrates probe-dependent activity, that is, it inhibits CXCL11- over CXCL10-mediated G protein activation with 12-fold selectivity. Together with a previously reported biased negative allosteric modulator from our group, the present study provides additional information on the molecular requirements for allosteric modulation of CXCR3.

## Introduction

Agonists of G protein-coupled receptors (GPCRs) do not uniformly activate all cellular signaling pathways linked to a given receptor (a phenomenon termed ligand or agonist bias).[1–3] The CXC chemokine receptor 3 (CXCR3), a group A GPCR, is an excellent example: its functions are regulated by at least three endogenous protein ligands, CXC chemokines CXCL9, CXCL10, and CXCL11. These chemokines are mainly induced by interferon (IFN)-γ.[4] Each of them evokes different cellular responses upon binding to the receptor.[4–7] Thus, ligand-biased signaling is employed by nature itself to regulate the functions of CXCR3. Dysregulation of the CXCR3 network is associated with a number of autoimmune diseases such as rheumatoid arthritis, psoriasis, and multiple sclerosis.[8,9] Several animal models showed beneficial effects of down-regulation of CXCR3 signaling in rheumatoid arthritis,[10] allograft rejection,[11] and in some types of cancer.[12,13] At the same time the role of CXCR3 is much more complex and controversial in neuroinflammation[14–16] and immune responses to bacterial infection.[17] The only clinical candidate that targets CXCR3, AMG487, failed in phase IIa clinical trials for rheumatoid arthritis and psoriasis due to lack of efficacy.[18,19]

The complex properties of allosteric modulation of GPCRs, which include probe dependence and biased signaling, can present both challenges and opportunities for preclinical drug discovery.[2,20–27] By blocking harmful and maintaining beneficial stimuli of the same endogenous signal transmitter, allosteric modulators provide finely tuned instruments for restoring physiological conditions affected by pathology. For CXCR3, two nonpeptidic small-molecule ligands were reported as biased allosteric agonists of the receptor, as they recruit β-arrestin with greater efficacy than the endogenous agonist.[28,29] One of them, PS372424, demonstrated beneficial therapeutic effects in a humanized mouse model of arthritis.[30]

From a pharmacological perspective, an agonist can not only elicit biased signaling, but antagonists can also act permissively.[31] Our research group recently reported the discovery of the first biased negative allosteric modulator of CXCR3 (boronic acid **14**, see Scheme 2 below), which preferentially blocks CXCL11-mediated β-arrestin recruitment over G protein activation.[32]

Another important aspect of allosteric modulation of chemokine receptors is probe dependence. Probe-dependent allostery provides a manner for fine tuning the chemokine response. For example, the antagonist of the chemokine receptor CXCR4, AMD-3100, blocks CXCL12 binding to CXCR4 but

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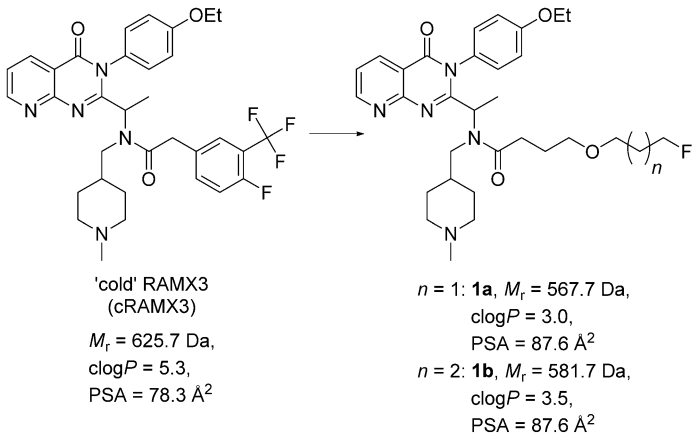
enhances the binding of CXCL12 to its other natural receptor, CXCR7.[33] Aplaviroc, an allosteric modulator of the chemokine receptor CCR5, alters its conformation, rendering it unable to bind and support HIV-1 viral fusion and the binding of [$^{125}$I]CCL3. At the same time it does not interfere with the binding and function of CCL5.[34,35] For CXCR3, probe dependence was reported for nonpeptidic small-molecule allosteric agonists.[28,29] These ligands are able to suppress the binding of CXCL10 to CXCR3, but not that of CXCL11.[29] Probe dependence can therefore lead to major therapeutic advantages in the pharmacological manipulation of chemokine systems.

Herein we report the discovery of small-molecule negative allosteric modulators of CXCR3 that show a bias for the inhibition of a given signaling pathway, and probe-dependent behavior. Among them, **1 b** is a unique negative allosteric modulator that demonstrates probe-dependent biased signaling at CXCR3; it has the propensity to inhibit a specific pathway depending on the endogenous agonist used.



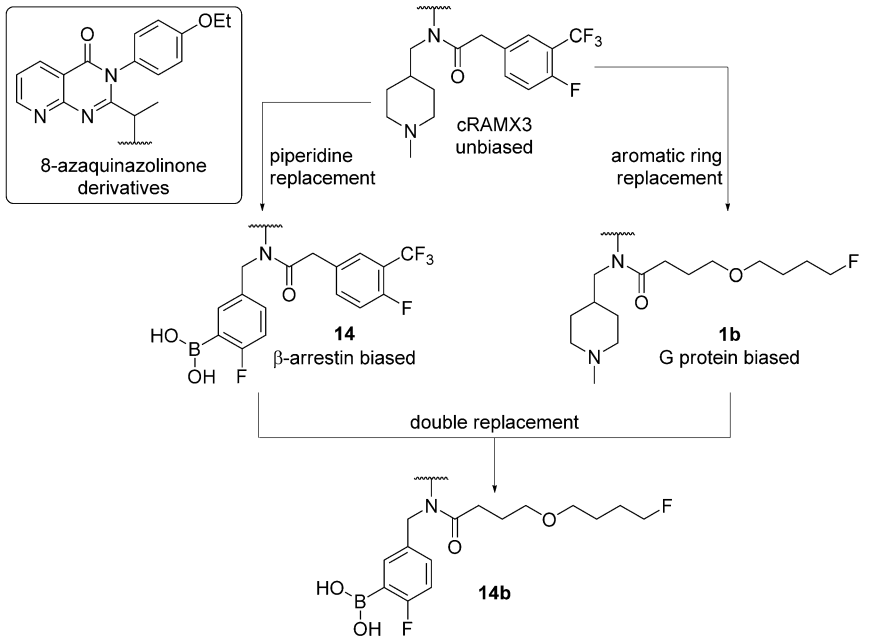

**Scheme 1.** Design of 8-azaquinazolinone chemotype CXCR3 ligands with improved physicochemical properties.

## Results and Discussion

### Ligand design

Alkyl-substituted 8-azaquinazolinones were originally identified as antagonists of the chemokine receptor CXCR3 by high-throughput screening.[36,37] Subsequent structure–activity relationship (SAR) studies have shifted the focus of medicinal chemists toward lead compounds with aromatic rings in place of alkyl chains.[37,38] Although this modification led to increases in binding affinity, the accompanying rigidification of the scaffold did not improve the physicochemical parameters of the ligands, which suffer high lipophilicity.

To facilitate the in vivo testing of these negative allosteric modulators we attempted to optimize their physicochemical properties by increasing their flexibility and the hydrophilicity of the carboxamide side chain. The presence of rigid hydrophobic aromatic rings is known to have a negative effect on the ADME properties of drug-like compounds.[39,40] Hence, we replaced the fluorinated aromatic ring in the structure of non-tritiated ('cold') allosteric radioligand RAMX3[41] (cRAMX3) with a flexible fluorooxoalkyl chain (Scheme 1). According to available SAR data, the optimal length of the alkyl side chain necessary for antagonistic effects is $C_{8-9}$.[37] To increase the hydrophilicity of the molecule while keeping its polar surface area (PSA) below the commonly used cutoff for good oral availability (< 90 Å$^2$), we introduced a single oxygen atom into the alkyl chain (compounds **1 a** and **1 b**, Scheme 1). Addition of the second oxygen atom to the alkyl chain would further improve hydrophilicity (from clog $P$ 3.0 to 2.62), but would lead to higher PSA (96.80 Å$^2$). Further elongation of the alkyl chain has been shown to have a minor influence on the compounds' bioactivity.[37] It would also negatively affect lipophilicity, increasing clog $P$ to 3.98 for the closest higher homologue of **1 b**. Therefore, we focused ligand design onto the identification of the shortest alkyl chain that would elicit activity in binding and functional assays at CXCR3.




**Scheme 2.** Design of 'chimeric' compound **14 b** through the combination of structural features of ligands biased either toward β-arrestin (**14**) or toward G protein (**1 b**).

The functionally selective boronic acid **14**[32] (Scheme 2) exhibits 24-fold selectivity for inhibiting CXCL11-mediated β-arrestin over G protein recruitment. After synthesizing **1a** and **1b** and confirming the high functional bias and probe dependence for the latter (see below) we became interested in testing the combination of structural features of these two molecules. The side chain of **1b** was thus introduced into the structure of **14**, leading to the design of the 'chimeric' ligand **14b** (Scheme 2).

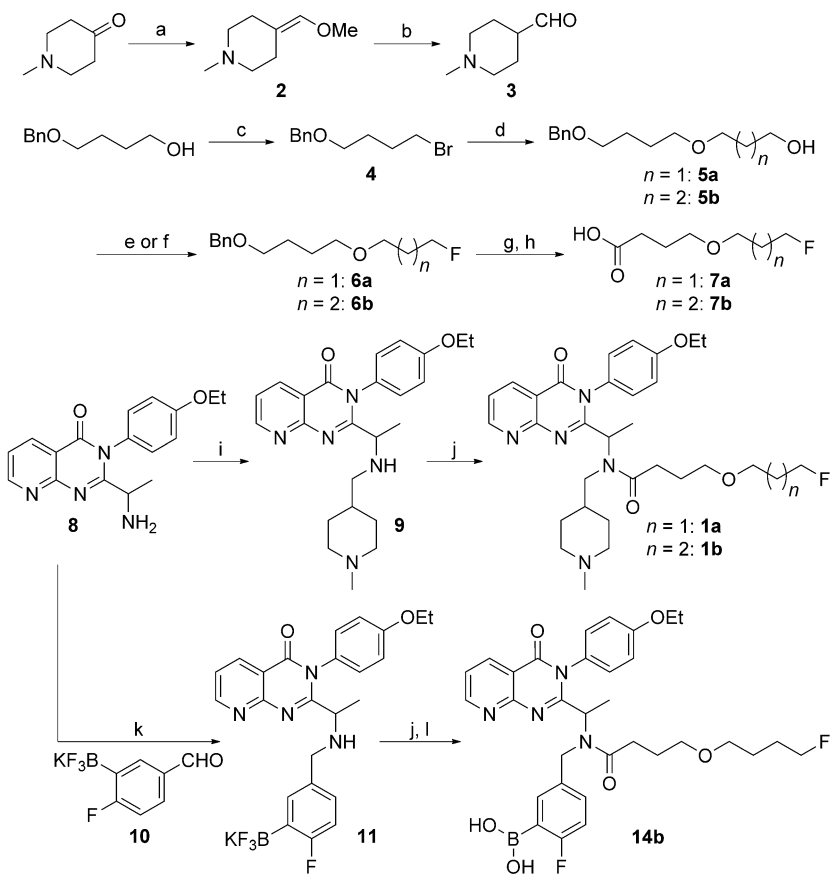
## Synthesis

Aldehyde **3** (Scheme 3) was synthesized according to a published two-step sequence:[42] a Wittig reaction between 1-methylpiperidin-4-one and (methoxymethyl)triphenylphosphonium chloride, followed by acidic hydrolysis of the resulting vinyl ether **2**.

The fluoroalkyloxycarboxylic acids **7a** and **7b** were synthesized through a five-step sequence: first, commercially available 4-(benzyloxy)butan-1-ol was converted into the corresponding bromide **4**. The bromine atom was then substituted by propane-1,3-diol or butane-1,4-diol to afford **5a** and **5b**, respectively. The free hydroxy group of **5a** was replaced by fluorine via treatment with diethylaminosulfur trifluoride, leading to **6a**. In contrast, application of this method to the synthesis of **6b** resulted in poor conversion of the starting material, and quenching of the reaction mixture with methanol yielded a high content of the corresponding methyl ether. Therefore, **6b** was instead prepared by a combination of tosylation and nucleophilic substitution. Finally, the benzyl protecting groups were removed by catalytic hydrogenation, and the resulting primary alcohols were converted into carboxylic acids **7a** and **7b** via Jones oxidation.

The synthesis of the racemic primary amine **8** was reported previously.[38,41] Reductive alkylation of **8** with piperidinealdehyde **3** was accomplished with sodium triacetoxyborohydride, and the resulting secondary amine **9** was coupled with in-situ-activated carboxylic acids **7a** and **7b**. The tertiary amides **1a** and **1b** obtained from this sequence had improved drug-like properties, with lower molecular weight ($M_r$ 567.7 and 581.7 Da, respectively, vs. 625.7 Da) and higher polarity ($t_R$ = 15.7 and 16.2 min, respectively, vs. 17.5 min in RP-HPLC) than cRAMX3.

For the synthesis of compound **14b**, primary amine **8** was reductively alkylated with aldehyde **10**[42] by using a pyridine–borane complex as reducing agent.[32] The resulting secondary amine **11**[32] was acylated with 4-(4-fluorobutoxy)butanoic acid (**7b**), and the trifluoroborate moiety was finally converted into a boronic acid by treatment with trimethylsilyl chloride in aqueous acetonitrile, yielding **14b**.




**Scheme 3.** Synthesis of building blocks and fluorooxoalkyl derivatives of 8-azaquinazolinones. *Reagents and conditions*: a) $Ph_3P^+CH_2OMeCl^-$, *t*BuOK, THF, −20 °C→RT, 18 h, 64%; b) 5 N HCl, $H_2O$/THF, 2 h, 54%; c) $CBr_4$, $PPh_3$, $CH_2Cl_2$, 0 °C→RT, 16 h, 91%; d) propane-1,3-diol or butane-1,4-diol, KOH, DMSO, 0 °C→RT, 2 h, 65–75%; e) DAST, $CH_2Cl_2$, −30 °C→RT, 5 h, 39% for $n$ = 2, 0% for $n$ = 1; f) TsCl, $NEt_3$, DMAP, $CH_2Cl_2$, 0 °C→RT, 3 h; then $nBu_4N^+F^-$, THF, reflux, 20 h, 65% for $n$ = 1; g) $H_2$, Pd/C, MeOH, 6 h; h) $CrO_3$, $H_2SO_4$, acetone, 30–40 min, 80–90% over two steps; i) **3**, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane, 20 h, 77%; j) **7a** or **7b**, HATU, DIPEA, DMF, 45 °C, 20 h, 65–72%; k) **10**, pyridine–borane complex, $KHF_2$, THF, RT, 16 h, 92%; l) $Me_3SiCl$, $H_2O$/MeCN, 2 h, 34% over two steps.

## Biological properties

To determine the binding affinities of novel allosteric modulators to the receptor, we used the allosteric radioligand RAMX3[41] displacement assay. The ability of novel allosteric modulators to inhibit the chemokine-mediated activation of CXCR3 was measured by a [$^{35}$S]GTPγS incorporation assay and a β-arrestin 2 recruitment assay (PathDetect, DiscoverX). The activation of CXCR3 was achieved by chemokines CXCL11 and CXCL10. The data were analyzed by nonlinear regression using the algorithms in Prism 5.0 (GraphPad Software,

San Diego, CA, USA), fitting experimental curves to the parameters of the tertiary complex model to determine the equilibrium dissociation constant $pK_b$ and cooperativity factors $\alpha$ and $\alpha\beta$ [Equations (1)–(6)].[27,32]

Compound **1b** exhibited good affinity in the radioligand displacement assay ($K_b = 81$ nM) and noncompetitive displacement of RAMX3 ($\alpha = 0.14$) (Table 1). In functional assay **1b** inhibited CXCL11-induced activation of G protein at a lower concentration than β-arrestin 2 recruitment (187-fold difference in $K_b$: 1.9 vs. 355 nM, with similar cooperativity factors $\alpha\beta$: 0.09 vs. 0.07, respectively; Tables 2 and 3, Figure 1). Additionally, for **1b** strong probe dependence was observed. Compound **1b** inhibited CXCL10-induced activation of G protein at a significantly higher concentration ($K_b$ value of 22.9 nM) relative to inhibition of the CXCL11-induced activation (two-tail unpaired *t*-test, $p < 0.05$). The cooperativity between **1b** and CXCL10 was also decreased ($\alpha\beta = 0.30$). In the β-arrestin 2 recruitment assay **1b** showed no probe dependence (Table 3, Figure 1); **1b** is thus a unique negative allosteric modulator that demonstrates probe-dependent biased signaling.

While **1b**, with nine heavy atoms in the carboxamide side chain, had good affinity and activity in the G protein activation assay, ligand **1a**, which is one methylene group shorter, exhibited greatly diminished binding and functional activity. This observation is in agreement with previous SAR data.[37]

**Table 1.** Affinity of 8-azaquinazoline derivatives as determined by allosteric radioligand RAMX3 displacement assay.[a]

| Compd | $pK_b$ (95% C.I.) | $K_b$ | $\alpha$ |
|---|---|---|---|
| cRAMX3 | 7.92 (8.10–7.75) | 11.9 | 0 |
| **1a** | 6.01 (6.30–5.71) | 980 | 0.21 |
| **1b** | 7.09 (7.37–7.05) | 81.1 | 0.14 |
| **14** | 7.42 (7.79–7.05) | 38.1 | 0.29 |
| **14b** | 5.67 (5.82–5.52) | 2120 | 0 |

[a] Allosteric radioligand displacement assays were carried out with RAMX3 (1 nM) and membrane preparations of HEK293T cells transiently expressing CXCR3; values are the mean with 95% confidence interval (C.I.) of three experiments performed in triplicate.

**Table 2.** Ability of 8-azaquinazolinone derivatives to inhibit CXCL10- and CXCL11-mediated activation of CXCR3 as measured in [$^{35}$S]GTPγS incorporation assays.[a]

| Compd | CXCL10 | | CXCL11 | |
|---|---|---|---|---|
| | $pK_b$ (95% C.I.) | $\alpha\beta$ | $pK_b$ (95% C.I.) | $\alpha\beta$ |
| cRAMX3 | 8.11 (8.44–7.77) | 0.01 | 7.84 (8.06–7.62) | 0 |
| **1a** | < 5.0 | – | 8.26 (8.93–7.59) | 0.44 |
| **1b** | 7.64* (8.06–6.86) | 0.30 | 8.73 (9.38–8.09) | 0.14 |
| **14** | 7.24* (7.58–6.90) | 0.05 | 6.05 (6.61–5.49) | 0.10 |
| **14b** | 6.97 (7.40–6.55) | 0.08 | 7.62 (8.22–7.03) | 0.31 |

[a] Inhibition of CXCL10- (50 nM) and CXCL11-mediated (5 nM) activation of CXCR3 was determined by [$^{35}$S]GTPγS incorporation assay with the membrane preparations of HEK293T cells transiently expressing CXCR3. Values reflect the mean $pK_b$ with 95% confidence interval (C.I.) of three to five experiments performed in triplicate. Two-tail unpaired *t*-test was performed to estimate the significance between $pK_b$ and $\alpha\beta$ values observed between both chemokines: $^*p < 0.05$.

We have previously reported **14** as a negative allosteric modulator that preferentially inhibits CXCL11-mediated β-arrestin 2 recruitment over G protein activation.[32] After discovering that allosteric modulator **1b** preferably blocks the CXCL11-mediated activation of G protein rather than β-arrestin recruitment, we designed boronic acid derivative **14b** by combining

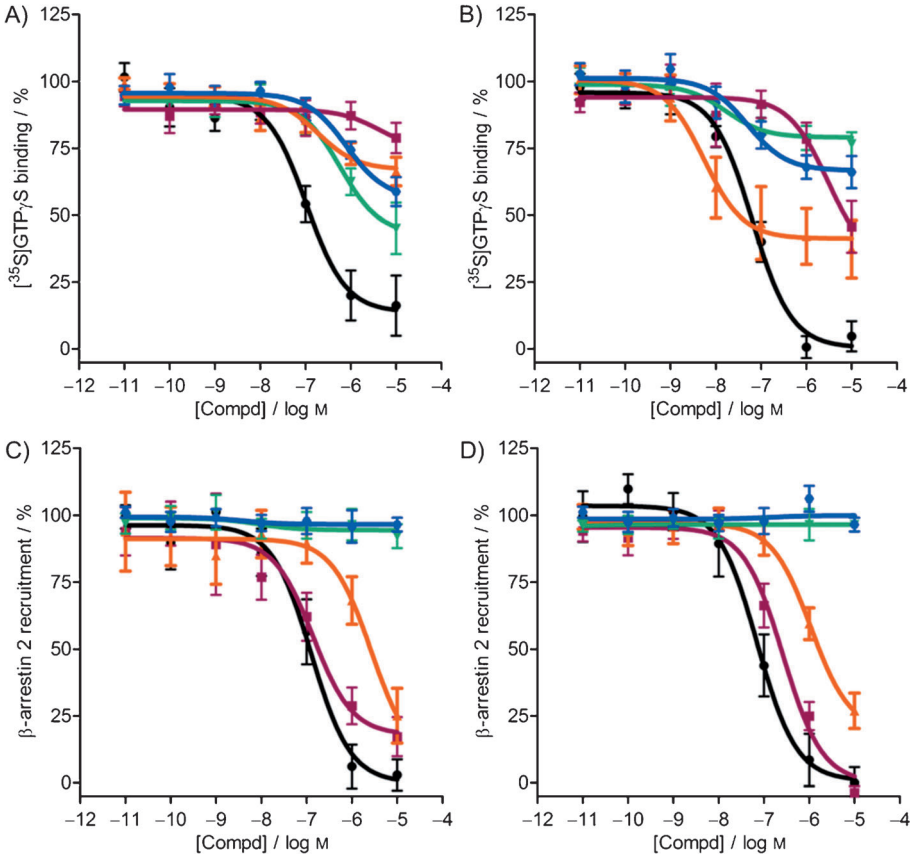



**Figure 1.** Ligand-biased and probe-dependent signaling in CXCR3. A), B): The ability of 8-azaquinazolinone derivatives to inhibit the CXCL10- (50 nM) and CXCL11-mediated (5 nM) activation of CXCR3 was determined by [$^{35}$S]GTPγS incorporation assay with membrane preparations of HEK293T cells transiently expressing CXCR3. C), D): The ability of 8-azaquinazolinone derivatives to inhibit the CXCL10- (400 nM) and CXCL11-mediated (100 nM) recruitment of β-arrestin 2 to CXCR3 was determined with HEK293 cells expressing all the components of PathHunter (DiscoverX) and CXCR3-PK2. Black: cRAMX3, green: **1a**, orange: **1b**, magenta: **14**, blue: **14b**.

**Table 3.** Ability of 8-azaquinazolinone derivatives to inhibit CXCL10- and CXCL11-mediated activation of CXCR3 as measured in β-arrestin 2 recruitment assays.[a]

| Compd | CXCL10 | | CXCL11 | |
|---|---|---|---|---|
| | $pK_b$ (95% C.I.) | $\alpha\beta$ | $pK_b$ (95% C.I.) | $\alpha\beta$ |
| cRAMX3 | 7.66 (7.99–7.33) | 0 | 7.68 (8.00–7.35) | 0 |
| **1a** | – | – | – | – |
| **1b** | 6.41 (6.96–5.87) | 0.02 | 6.45 (6.74–6.16) | 0.07 |
| **14** | 7.68 (8.13–7.23) | 0.04 | 7.12 (7.33–6.91) | 0 |
| **14b** | – | – | – | – |

[a] Inhibition of CXCL10- (400 nM) and CXCL11-mediated (100 nM) recruitment of β-arrestin 2 to CXCR3 was determined with HEK293 cells expressing all the components of PathHunter (DiscoverX) and CXCR3-PK2. Values reflect the mean $pK_b$ with 95% confidence interval (C.I.) of three experiments performed in triplicate. Two-tail unpaired *t*-test was performed to estimate the significance between $pK_b$ and $\alpha\beta$ values observed between both chemokines.

structural features of **1b** and **14**. The 'chimeric' ligand **14b** displayed greatly decreased affinity for CXCR3 in RAMX3 displacement and both functional assays (Table 1–3, Figure 1). At the same time, double replacement of substituents at tertiary amide core of cRAMX3 restored the competitive character of the radioligand displacement ($\alpha = 0$; Figure 1, Table 1).

These observations support our hypothesis that structurally similar allosteric modulators can bind to CXCR3 in different orientations.[32] Based on the present data we hypothesize further that these orientations elicit different functional responses, leading to biased signal transduction. It is plausible that by optimization of the high-affinity ligand cRAMX3 we forced **1b** (and earlier **14**)[32] to adopt functionally selective orientation, whereas by combining structural modifications we lost this binding preference, erasing the signaling bias and impairing affinity (**14b**).

## Conclusions

Regulation of CXCR3 signaling is an attractive therapeutic strategy in autoimmune diseases. However, the complex pharmacology of the endogenous chemokine signaling system poses a nontrivial challenge for the design of compounds with tailored functional activity. Herein we describe the first negative allosteric modulator of CXCR3 with functional selectivity to inhibit the G protein pathway (**1b**). Additionally, the compound demonstrated probe-dependent functional bias and better physicochemical properties (molecular weight and polarity) than known ligands of the same chemotype. Such information is crucial for further structure-based development of allosteric ligands that target CXCR3, particularly of those intended for in vivo applications.

## Experimental Section

### *Chemistry*

**General**: All chemicals and solvents were purchased from Sigma–Aldrich, Acros, or Alfa Aesar and were used without additional purification. Anhydrous solvents were of the highest commercially available grade and were stored over molecular sieves under a nitrogen atmosphere. Flash chromatography was performed on Merck silica gel 60 (40–63 μm) as stationary phase under positive pressure of dry nitrogen gas. HR-ESIMS analyses were conducted on a Bruker Daltonik microTOF II ($M/\Delta M > 16500$). HPLC–MS and HPLC purity analyses were performed with an Agilent Binary Gradient system using UV detection ($\lambda = 254$ nm) in combination with ChemStation software (Rev. A. 10.02 [1757]). A Zorbax Eclipse XDB-$C_8$ (4.6 mm × 150 mm, 5 μm) column was used at a flow rate of 0.5 mL min$^{-1}$ in reversed-phase mode (eluent: MeOH/$H_2O$ + 0.1% HCOOH, 10→100% in 21 min, 100% for 3 min). Mass detection was conducted with a Bruker Esquire 2000 ion-trap mass spectrometer using an APCI or ESI ionization source. $^1$H and $^{13}$C NMR spectra were recorded on Bruker Avance 360 or 600 FT-NMR spectrometers. Chemical shifts (ppm) were calculated relative to TMS ($^1$H) or solvent signal ($^{13}$C) as internal standards. Synthesis of previously reported intermediates are provided in the Supporting Information.

**3-[4-(Benzyloxy)butoxy]propan-1-ol (5a)**: To a slurry of propane-1,3-diol (1.62 g, 21.3 mmol) and KOH (1.61 g, 28.7 mmol) in DMSO (10 mL) [(4-bromobutoxy)methyl]benzene (**4**) (1.03 g, 4.2 mmol) was added dropwise at 0 °C under argon. The mixture was stirred for 20 h at 0 °C. The reaction mixture was then diluted with $H_2O$ (20 mL), the layers were separated, and the organic phase was diluted with $CH_2Cl_2$ (10 mL) and washed with saturated aqueous $NH_4Cl$, $NaHCO_3$, and NaCl. The organic layer was dried over anhydrous $Na_2SO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (1→3% MeOH in $CH_2Cl_2$) and obtained as a colorless oil (0.66 g, 65%): HPLC–MS: $t_R = 23.3$ min, *m/z* (ESI) found 261.5, calcd 261.3 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (360 MHz, $CDCl_3$): $\delta =$ 7.26–7.37 (m, 5H), 4.50 (s, 2H), 3.75 (q, $J = 5.4$ Hz, 2H), 3.59 (t, $J =$ 5.7 Hz, 2H), 3.39–3.52 (m, 4H), 2.47 (t, $J = 5.3$ Hz, 1H), 1.81 (quint., $J = 5.6$ Hz, 2H), 1.67 ppm (m, $J = 2.9$ Hz, 4H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta = 138.6$, 128.3 (2C), 127.6 (2C), 127.5, 72.8, 71.0, 70.1, 70.0, 62.1, 32.0, 26.4, 26.4 ppm; HR-ESIMS: *m/z* found 261.1462, calcd 261.1461 for $C_{14}H_{22}NaO_3$ $[M+Na]^+$.

**4-[4-(Benzyloxy)butoxy]butan-1-ol (5b)**: To a slurry of butane-1,4-diol (2.87 g, 31.9 mmol) and KOH (2.68 g, 47.8 mmol) in DMSO (25 mL) [(4-bromobutoxy)methyl]benzene (**4**) (1.55 g, 6.37 mmol) was slowly added under nitrogen atmosphere under gentle cooling in an ice bath (maintaining DMSO liquid). The mixture was allowed to warm to ambient temperature and stirred for 2 h. Then the reaction was quenched with $H_2O$ (30 mL) and the two layers were separated. The organic phase was diluted with $CH_2Cl_2$ (15 mL) and washed with saturated aqueous $NH_4Cl$ and $NaHCO_3$. The organic layer was dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (2.5% MeOH in $CH_2Cl_2$), yielding **5b** as a colorless liquid (1.2 g, 75%): TLC (2.5% MeOH in $CH_2Cl_2$): $R_f = 0.22$; HPLC–MS: $t_R =$ 23.8 min, *m/z* (ESI) found 275.6, calcd 275.3 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (600 MHz, $CDCl_3$): $\delta = 7.30$–7.36 (m, 4H), 7.26–7.30 (m, 1H), 4.50 (s, 2H), 3.63 (br s, 2H), 3.47–3.52 (m, 2H), 3.42–3.47 (m, 4H), 2.52 (br s, 1H), 1.61–1.72 ppm (m, 8H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta = 138.6$, 128.3 (2C), 127.6 (2C), 127.5, 72.8, 70.8, 70.8, 70.1, 62.7, 30.4, 26.9, 26.5, 26.4 ppm; HR-ESIMS: *m/z* found 253.1799, calcd 253.1798 for $C_{15}H_{25}O_3$ $[M+H]^+$.

**{[4-(3-Fluoropropoxy)butoxy]methyl}benzene (6a)**: A 50 mL Schlenk flask was charged with **5a** (0.34 g, 1.42 mmol) in $CH_2Cl_2$ (20 mL) to give a colorless solution. Triethylamine (0.99 mL, 7.1 mmol) and 4-dimethylaminopyridine (DMAP; 18 mg, 0.15 mmol) were added, and the mixture was cooled to 0 °C. Tolue-

nesulfonyl (tosyl) chloride (0.41 g, 2.1 mmol) was added. The mixture was stirred for 10 min at 0 °C and was then allowed to warm to ambient temperature over 3 h. Consumption of starting material was monitored by TLC (40% $Et_2O$ in hexane). Then the reaction mixture was diluted to 50 mL with $CH_2Cl_2$ and washed with $H_2O$ (2×20 mL) and brine (30 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The resulting residue was dried under high vacuum. The resulting crude tosylate was dissolved in anhydrous MeCN (15 mL), and a 1 M solution of tetra-*n*-butylammonium fluoride (4.25 mL, 4.25 mmol) was added. The resulting mixture was held at reflux for 20 h, then cooled to ambient temperature and concentrated under reduced pressure. The residue was partitioned between $Et_2O$ (25 mL) and $H_2O$ (25 mL). The aqueous layer was discarded and the organic layer was washed with saturated aqueous $NaHCO_3$ (20 mL) and brine (20 mL), then dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (10% $Et_2O$ in hexane), yielding **6a** as a colorless liquid (218 mg, 64%): TLC (35% $Et_2O$ in hexane): $R_f = 0.54$; HPLC–MS: $t_R =$ 24.4 min, *m/z* (ESI) found 263.5, calcd 263.3 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (600 MHz, $CDCl_3$): $\delta = 7.30–7.37$ (m, 4H), 7.25–7.30 (m, 1H), 4.50 (s, 2H), 4.07 (ddt, $J = 56.5$, 10.2, 6.2 Hz, 2H), 3.45–3.52 (m, 4H), 3.42 (t, $J = 6.1$ Hz, 2H), 1.91 (quint., $J =$ 6.2 Hz, 2H), 1.60–1.71 ppm (m, 4H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta =$ 138.6, 128.3 (2C), 127.6 (2C), 127.5, 81.3 (d, $J = 164.2$ Hz), 72.8, 70.8, 70.1, 66.3 (d, $J = 5.6$ Hz), 30.9 (d, $J = 19.8$ Hz), 26.5, 26.4 ppm; HR-ESIMS: *m/z* found 241.1600, calcd 241.1598 for $C_{14}H_{22}FO_2$ $[M+H]^+$.

**{[4-(4-Fluorobutoxy)butoxy]methyl}benzene (6b)**: A solution of **5b** (0.30 g, 1.19 mmol) in anhydrous $CH_2Cl_2$ (10 mL) was purged with dry nitrogen gas while cooling to −30 °C followed by addition of diethylaminosulfur trifluoride (DAST; 0.31 mL, 2.4 mmol). The reaction mixture was stirred for 5 h and was then quenched with MeOH (20 mL). The mixture was allowed to warm to ambient temperature. The solvent was removed under reduced pressure and the pale-yellow liquid residue was dried in high vacuum. The residue was purified by flash chromatography (15→20% $Et_2O$ in hexane), yielding **6b** as colorless oil (119 mg, 39%): HPLC–MS: $t_R =$ 25.1 min, *m/z* (ESI) found 277.5, calcd 277.3 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (360 MHz, $CDCl_3$): $\delta = 7.25–7.42$ (m, 5H), 4.50 (s, 2H), 4.45 (dt, $J = 47.3$, 6.0 Hz, 2H), 3.49 (t, $J = 6.1$ Hz, 2H), 3.34–3.46 (m, 4H), 1.59–1.86 ppm (m, 8H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta = 138.6$, 128.3 (2C), 127.6 (2C), 127.5, 84.0 (d, $J =$ 164.2 Hz), 72.8, 70.6, 70.1, 70.1, 27.3 (d, $J = 19.8$ Hz), 26.5, 26.5, 25.6 ppm (d, $J = 5.3$ Hz); HR-ESIMS: *m/z* found 277.1579, calcd 277.1574 for $C_{15}H_{23}FNaO_2$ $[M+Na]^+$.

**4-(3-Fluoropropoxy)butanoic acid (7a)**: To a slurry of palladium on carbon (10 wt%, 25 mg, 0.024 mmol) in anhydrous THF (5 mL) was added **6a** (0.22 g, 0.91 mmol) followed by a rinse with THF (1.5 mL). The slurry was stirred under hydrogen atmosphere for 20 h. The reaction mixture was filtered over a Celite plug and the filter cake was washed with EtOAc (15 mL). The filtrate was concentrated under reduced pressure and dried under high vacuum yielding the crude fluoroalcohol as a colorless oil. This was dissolved in acetone (3 mL), and Jones reagent was added until a brown–orange color persisted (total amount ~1.5 mL). The mixture was stirred at ambient temperature for 3 h. Then the reaction was quenched with *i*PrOH (10 mL) and diluted with 1 N HCl to dissolve precipitated salts (25 mL). The aqueous layer was extracted with EtOAc (3×25 mL). The combined organic layers were washed with brine (15 mL), dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (AcOH/EtOAc/EtOH/hexane 0.25:9.25:3:87.5), yielding **7a** as a pale-yellow oil (134 mg, 90% over two steps): HPLC–MS: $t_R = 15.3$ min, *m/z* (ESI) found 187.3, calcd 187.2 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (600 MHz, $CDCl_3$): $\delta =$ 11.20 (br s, 1H), 4.53 (dt, $J = 47.2$, 5.9 Hz, 2H), 3.54 (t, $J = 6.1$ Hz, 2H), 3.49 (t, $J = 6.0$ Hz, 2H), 2.46 (t, $J = 7.3$ Hz, 2H), 1.94 (dquint., $J = 25.7$, 6.0 Hz, 2H), 1.91 ppm (tt, $J = 7.2$, 6.2 Hz, 2H); $^{13}$C NMR (151 MHz, $CDCl_3$): $\delta = 179.3$, 81.0 (d, $J = 163.6$ Hz), 69.7, 66.4 (d, $J =$ 5.5 Hz), 30.9, 30.8 (d, $J = 19.8$ Hz), 24.7 ppm; HR-ESIMS: *m/z* found 187.0750, calcd 187.0741 for $C_7H_{13}FNaO_3$ $[M+Na]^+$.

**4-(4-Fluorobutoxy)butanoic acid (7b)**: A solution of **6b** (110 mg, 0.43 mmol) and palladium on carbon (10 wt%, 11 mg, 10.34 μmol) in MeOH (2 mL) was stirred under hydrogen atmosphere for 20 h. The reaction mixture was filtered over a Celite plug and concentrated under reduced pressure. The crude fluoroalcohol (62 mg, 88%) was obtained as a pale-yellow oil. The crude product was dissolved in acetone (2 mL), and Jones reagent was added at 0 °C until a dark-orange color persisted. The reaction mixture was allowed to warm to ambient temperature over 40 min and was then quenched with *i*PrOH (10 mL). The mixture was additionally stirred for 25 min at ambient temperature, was then diluted with $H_2O$ (10 mL) and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine (10 mL), dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The crude product was obtained as a colorless oil (62 mg, 80% over two steps) after drying under high vacuum. The product **7b** was used for acylation of the secondary amines without further purification. HPLC–MS: $t_R = 18.5$ min, *m/z* (ESI) found 201.3, calcd 201.2 ($[M+Na]^+$), purity: n/a (254 nm, 220 nm); $^1$H NMR (600 MHz, $CDCl_3$): $\delta = 11.07$ (br s, 1H), 4.46 (dt, $J = 47.4$, 6.0 Hz, 2H), 3.46 (t, $J =$ 6.4 Hz, 2H), 3.47 (t, $J = 6.2$ Hz, 2H), 2.46 (t, $J = 7.4$ Hz, 2H), 1.90 (tt, $J = 7.4$, 6.4 Hz, 2H), 1.72–1.83 (m, 2H), 1.64–1.72 ppm (m, 2H); $^{13}$C NMR (151 MHz, $CDCl_3$): $\delta = 179.2$, 83.9 (d, $J = 163.6$ Hz), 70.3, 69.5, 31.0, 27.3 (d, $J = 19.8$ Hz), 25.5 (d, $J = 5.5$ Hz), 24.8 ppm; HR-ESIMS: *m/z* found 201.0903, calcd 201.0897 for $C_8H_{15}FNaO_3$ $[M+Na]^+$.

**3-(4-Ethoxyphenyl)-2-(1-{[(1-methylpiperidin-4-yl)methyl]amino}ethyl)pyrido[2,3-*d*]pyrimidin-4(3*H*)-one (9)**: To a suspension of primary amine **8**[41] (1.09 g, 3.5 mmol) in anhydrous 1,2-dichloroethane (10 mL) aldehyde **3** (0.45 g, 3.5 mmol) was added followed by sodium triacetoxyborohydride (1.1 g, 5.2 mmol) and acetic acid (0.2 mL, 3.5 mmol). The solution was stirred for 18 h at ambient temperature. Then the reaction mixture was diluted with $CH_2Cl_2$ (20 mL) and washed with saturated aqueous $NaHCO_3$. The organic layer was extracted with 1 N HCl (3×15 mL), the combined aqueous layers were basified with 25% aqueous $NH_3$ and back-extracted with EtOAc (3×20 mL). The combined organic layers were dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (eluent: $NH_3$(aq)/MeOH/$CH_2Cl_2$ 1:9:90). Yield: 1.14 g (77%) as a pale-yellow foam: HPLC–MS: $t_R = 10.7$ min, *m/z* (APCI) found 422.4, calcd 422.2 ($[M+H]^+$), purity: 95% (254 nm); $^1$H NMR (600 MHz, $CDCl_3$): $\delta = 8.99$ (dd, $J = 4.6$, 2.2 Hz, 1H), 8.61 (dd, $J = 7.8$, 2.2 Hz, 1H), 7.43 (dd, $J = 7.8$, 4.6 Hz, 1H), 7.14 (s, 2H), 7.03–7.09 (m, 2H), 4.11 (q, $J = 7.0$ Hz, 2H), 3.49 (q, $J = 6.6$ Hz, 1H), 2.83 (br d, $J =$ 11.2 Hz, 2H), 2.41 (dd, $J = 11.2$, 6.7 Hz, 1H), 2.25 (s, 3H), 2.20 (dd, $J = 11.2$, 6.7 Hz, 1H), 1.88 (t, $J = 11.7$ Hz, 2H), 1.65–1.77 (m, 2H), 1.47 (t, $J = 7.0$ Hz, 3H), 1.30 (d, $J = 6.7$ Hz, 3H), 1.27–1.34 (m, 1H), 1.16–1.27 ppm (m, 2H); $^{13}$C NMR (91 MHz, $CDCl_3$): $\delta = 165.5$, 162.9, 159.8, 157.8, 156.2, 136.8, 129.1, 129.1, 128.1, 122.2, 116.1, 115.9, 115.7, 63.9, 55.7, 55.6, 55.6, 53.9, 46.3, 36.1, 30.7, 30.5, 21.2, 14.7 ppm; HR-ESI-MS: *m/z* found 422.2560, calcd 422.2551 for $C_{24}H_{32}N_5O_2$ $[M+H]^+$.

***N*-{1-[3-(4-Ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]ethyl}-4-(4-fluorobutoxy)-*N*-[(1-methylpiperidin-4-yl)methyl]butanamide (1b)**: In a 50 mL Schlenk flask crude **7b** (32 mg, 0.18 mmol), HATU (75 mg, 0.19 mmol) and DIPEA (35 μL, 0.20 mmol) were dissolved in anhydrous DMF (1 mL) under dry nitrogen atmosphere. The mixture was stirred for 10 min followed by addition of **9** (49 mg, 0.116 mmol) as solution in anhydrous DMF (1 mL). The mixture was stirred for 20 h at 45 °C. The reaction mixture was diluted to 30 mL with $CH_2Cl_2$ and washed with saturated aqueous $NaHCO_3$ (2×15 mL) and brine (15 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (25% $NH_3$(aq)/MeOH/$CH_2Cl_2$ 1:9:90), yielding **1b** as a white foam (44 mg, 65%): HPLC–MS: $t_R$ = 16.2 min, *m/z* (APCI) found 582.6, calcd 582.7 ($[M+H]^+$), purity: 98% (254 nm); $^1$H NMR (360 MHz, $CDCl_3$, *two rotamers in ratio 1:0.35, shifts of the major rotamer are listed*): $\delta$ = 8.97 (dd, *J* = 4.7, 2.0 Hz, 1H), 8.59 (dd, *J* = 7.8, 2.0 Hz, 1H), 7.53 (dd, *J* = 8.6, 2.5 Hz, 1H), 7.42 (dd, *J* = 7.9, 4.6 Hz, 1H), 7.12–7.18 (m, 1H), 6.98–7.09 (m, 2H), 5.22 (q, *J* = 7.1 Hz, 1H), 4.47 (dt, *J* = 47.2, 6.1 Hz, 2H), 4.10 (q, *J* = 7.0 Hz, 2H), 3.33–3.59 (m, 6H), 2.86 (s, 2H), 2.32–2.51 (m, 2H), 2.27 (s, 3H), 1.76–1.87 (m, 4H), 1.61–1.75 (m, 7H), 1.46 (t, *J* = 7.0 Hz, 3H), 1.46 (d, *J* = 7.0 Hz, 3H), 1.21–1.38 ppm (m, 2H); $^{13}$C NMR (151 MHz, $CDCl_3$, *two rotamers in ratio 1:0.35, signals of the major rotamer are listed*): $\delta$ = 174.3, 163.0, 162.7, 159.7, 157.4, 156.1, 136.8, 129.7, 129.0, 127.9, 122.3, 116.4, 115.7, 115.3, 83.9 (d, *J* = 164.1 Hz), 70.1, 69.8, 63.8, 55.6 (2C), 52.9, 50.7, 46.2, 37.1, 30.6, 30.3, 30.2, 27.4 (d, *J* = 19.8 Hz), 25.6 (d, *J* = 5.5 Hz), 25.5, 16.8, 14.8 ppm; HR-ESIMS: *m/z* found 582.3456; calcd 582.3450 for $C_{32}H_{45}FN_5O_4$ $[M+H]^+$.

***N*-{1-[3-(4-Ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]ethyl}-4-(3-fluoropropoxy)-*N*-[(1-methylpiperidin-4-yl)-methyl]butanamide (1a)**: A solution of **7a** (96 mg, 0.59 mmol), HATU (238 mg, 0.63 mmol) and DIPEA (112 μL, 0.64 mmol) in anhydrous DMF (2 mL) was stirred for 10 min at ambient temperature under nitrogen atmosphere. A solution of **9** (165 mg, 0.39 mmol) in anhydrous DMF (1 mL) was added. The reaction mixture was stirred for 20 h at 45 °C. The reaction mixture was diluted with EtOAc to 30 mL, washed with saturated aqueous $NaHCO_3$ (15 mL) and brine (15 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. Residual DMF was removed under high vacuum. The crude product was purified by flash chromatography (25% $NH_3$(aq)/MeOH/$CH_2Cl_2$1:9:90). Yield: 160 mg, 72%; HPLC–MS: $t_R$ = 19.5 min, *m/z*(ESI) found 569.0, calcd 568.7 ($[M+H]^+$), purity: 99% (254 nm, 220 nm); $^1$H NMR (360 MHz, $CDCl_3$, *two rotamers in ratio 1:0.6, signals of the major rotamer are listed*): $\delta$ = 8.92 (dd, *J* = 4.6, 2.0 Hz, 1H), 8.59 (dd, *J* = 7.8, 2.0 Hz, 1H), 7.61 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.43 (dd, *J* = 7.8, 4.7 Hz, 1H), 7.16 (dd, *J* = 8.6, 2.6 Hz, 1H), 7.00–7.11 (m, 2H), 5.10 (q, *J* = 7.1 Hz, 1H), 4.52 (dt, *J* = 47.2, 6.0 Hz, 2H), 4.10 (q, *J* = 7.0 Hz, 2H), 3.44–3.56 (m, 3H), 3.40 (t, *J* = 6.0 Hz, 2H), 3.27–3.36 (m, 1H), 2.76–2.90 (m, 1H), 2.70 (s, 3H), 2.50–2.61 (m, 1H), 2.42 (t, *J* = 7.3 Hz, 2H), 1.92 (dquint., *J* = 26.1, 6.0 Hz, 2H), 1.82 (quint., *J* = 6.5 Hz, 2H), 1.76–1.87 (m, 2H), 1.65–1.74 (m, 2H), 1.51–1.59 (m, 1H), 1.46 (t, *J* = 6.9 Hz, 3H), 1.44 (d, *J* = 7.1 Hz, 3H), 1.30–1.41 (m, 1H), 0.79–1.05 ppm (m, 1H); $^{13}$C NMR (151 MHz, $CDCl_3$, *two rotamers, signals of the major rotamer are listed*): $\delta$ = 174.1, 162.9, 162.9, 159.8, 157.3, 156.0, 137.0, 129.9, 129.1, 127.8, 122.4, 116.5, 115.7, 115.3, 81.4 (d, *J* = 163.0 Hz), 69.7, 66.3 (d, *J* = 5.5 Hz), 63.8, 55.7, 55.5, 53.6, 49.3, 45.0 (brs), 35.6, 30.8 (d, *J* = 19.8 Hz), 30.3, 28.6 (brs), 27.9 (brs), 25.4, 16.5, 14.8 ppm; HR-ESIMS: *m/z* found 568.3289; calcd 568.3294 for $C_{31}H_{43}FN_5O_4$ $[M+H]^+$.

**{5-[(*N*-{1-[3-(4-Ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]ethyl}-4-(4-fluorobutoxy)butanamido)methyl]-2-fluorophenyl}boronic acid (14b)**: A solution of HATU (189 mg, 0.50 mmol), **7b** (84 mg, 0.47 mmol), and DIPEA (95 μL, 0.54 mmol) in anhydrous DMF (1 mL) was stirred for 10 min at ambient temperature under nitrogen atmosphere. A solution of potassium (5-[({1-[3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-*d*]pyrimidin-2-yl]ethyl}amino)methyl]-2-fluorophenyl}trifluoroborate (**11**)[32] (124 mg, 0.24 mmol) in anhydrous DMF (1 mL) was added and the reaction mixture was stirred for 20 h at 45 °C under nitrogen atmosphere. The same amount of activated 4-(4-fluorobutoxy)butanoic acid was added and stirring was continued for another 24 h. Then the reaction mixture was diluted with EtOAc (15 mL) and washed with saturated $NaHCO_3$ (2×10 mL) and brine (10 mL). The organic layer was dried over anhydrous $Na_2SO_4$, filtered and concentrated under reduced pressure. The residue was redissolved in the mixture of MeCN and $H_2O$ (3:1, 10 mL) and treated with chlorotrimethylsilane (0.30 mL, 2.36 mmol). The mixture was stirred at ambient temperature for 2 h. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography (2.5% MeOH in $CH_2Cl_2$), yielding **14b** as a pale-yellow oil (50 mg, 34%): HPLC–MS: $t_R$ = 19.7 min, *m/z* (APCI) found 623.5, 637.4, calcd 623.5 ($[M+H]^+$), 637.5 ($[M+CH_3]^+$), purity: 95% (254 nm); $^1$H NMR (600 MHz, $CDCl_3$, *two rotamers in ratio 1:0.07, signals of the major rotamer are listed*): $\delta$ = 9.00 (dd, *J* = 4.6, 1.9 Hz, 1H), 8.66 (dd, *J* = 7.8, 1.8 Hz, 1H), 8.08 (d, *J* = 3.9 Hz, 1H), 7.98 (brs, 2H), 7.79 (dd, *J* = 8.7, 2.6 Hz, 1H), 7.49 (dd, *J* = 7.8, 4.7 Hz, 1H), 7.15 (ddd, *J* = 11.3, 8.7, 2.8 Hz, 2H), 7.06–7.11 (m, 1H), 6.98–7.05 (m, 2H), 5.21 (d, *J* = 18.8 Hz, 1H), 5.11 (q, *J* = 7.2 Hz, 1H), 5.12 (d, *J* = 18.9 Hz, 1H), 4.34 (dtd, *J* = 47.5, 6.1, 6.1, 1.7 Hz, 2H), 4.13 (q, *J* = 6.9 Hz, 2H), 3.29–3.35 (m, 4H), 2.50 (dt, *J* = 16.0, 7.3 Hz, 1H), 2.29 (dt, *J* = 16.1, 7.4 Hz, 1H), 1.83 (quint., *J* = 6.6 Hz, 2H), 1.50–1.70 (m, 4H), 1.48 (t, *J* = 6.9 Hz, 3H), 1.38 ppm (d, *J* = 7.3 Hz, 3H); $^{13}$C NMR (151 MHz, $CDCl_3$, *two rotamers, signals of the major rotamer are listed*): $\delta$ = 174.9, 166.8 (d, *J* = 245.9 Hz), 165.3, 162.4, 159.9, 157.1, 155.3, 137.8, 134.5 (d, *J* = 8.2 Hz), 134.1 (d, *J* = 3.0 Hz), 130.3, 129.1, 129.0, 127.7, 122.4, 116.7, 115.9, 115.5 (d, *J* = 25.8 Hz), 115.3, 83.8 (d, *J* = 164.1 Hz), 69.9, 69.5, 63.8, 53.5, 48.1, 30.3, 27.2 (d, *J* = 19.8 Hz), 25.4 (d, *J* = 5.2 Hz), 24.9, 16.2, 14.8 ppm; HR-ESIMS: *m/z* found 659.2812; calcd 659.2823 for $C_{33}H_{39}BF_2N_4O_6$ $[M+CH_2+Na]^+$.

### *Biological characterization*

*Cell culture and transfection*: Human embryonic kidney (HEK) cells were cultured in 150 mm cell culture plates in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin–streptomycin and incubated at 37 °C in a humid atmosphere with 5% $CO_2$. At 50–70% confluency, the cells were transiently transfected with 20 μg of the CXCR3 cDNA using TransIT-293 transfection reagent (Mirus Corporation) and harvested 48 h after transfection.

*Membrane preparation*: 48 h post-transfection, cells were washed with PBS twice and harvested with a scraper. Afterward cells were treated with Tris-EDTA buffer (10 mM Tris, 0.5 mM EDTA, 5 mM KCl, 140 mM NaCl, pH 7.4), and harvested using a cell scraper. Cells were pelleted at 1100 *g* for 8 min at 4 °C, re-suspended in Tris-EDTA-$MgCl_2$ buffer (50 mM Tris, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, pH 7.4) and followed by lysis with an Ultra-Turrax instrument. After centrifugation at 50000 *g* at 4 °C for 18 min, the membranes were re-suspended in binding buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$) and subsequently homogenized with a glass–Teflon homogenizer (20 strokes). The homogenized membranes were shock-frozen in liquid nitrogen and stored

at −80 °C. The protein concentration was determined by the Lowry method with bovine serum albumin (BSA) as a standard.[43]

*Allosteric radioligand displacement assays*: Receptor binding studies were performed on membrane preparations of HEK cells expressing the corresponding receptor. Tritium-labeled RAMX3 (specific activity: 80.4 Ci mmol$^{-1}$) at 1 nM was used for the assays. To determine unspecific binding, NBI-74330 (5 μM) was used. The assays were carried out in 96-well plates at a protein concentration of 30 μg mL$^{-1}$ in a total volume of 200 μL. The incubation buffer contained 20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, and 0.1% BSA (pH 7.4). After incubating for 1 h at 37 °C, the binding was stopped by filtration through Whatman GF/B filters using a 96-channel cell harvester (Brandel, Unterföhring). The filters were rinsed five times with ice-cold Tris-NaCl buffer. After drying for 3 h at 60 °C, filters were sealed with melt-on scintillator sheets (Melti-Lex G/HS) and the trapped radioactivity was measured in a microplate scintillation counter (Micro Beta Trilux scintillator). Three to five experiments per compound were performed with each concentration in triplicate. The $K_d$ and $B_{max}$ values were determined in the homologous competition assay and yielded following results: the p$K_d$ and $B_{max}$ values for the CXCR3 were 7.84 ± 0.09 and 5710 ± 700 fmol mg$^{-1}$. The p$K_d$ value determined in these experiments was used in the analysis of the data obtained in the allosteric radioligand binding studies to determine the parameters according to the allosteric ternary complex model.

*[$^{35}$S]GTPγS incorporation assays*: The [$^{35}$S]GTPγS incorporation assay was performed on membrane preparations of transiently transfected HEK293 cells expressing the CXCR3 receptor. The assay was carried out in 96-well plates at a final volume of 200 μL. The incubation buffer contained 20 mM HEPES, 10 mM $MgCl_2 \cdot 6H_20$, 100 mM NaCl, and 100 mg L$^{-1}$ saponin (pH 7.4). Membranes (30 μg mL$^{-1}$ of membrane protein), with 5 nM CXCL11 and various concentrations of test compound and 1 μM GDP were pre-incubated in the absence of [$^{35}$S]GTPγS for 30 min at 37 °C. After the addition of 0.10 nM [$^{35}$S]GTPγS membranes were incubated for additional 30 min at 37 °C. Incubation was terminated by filtration through Whatman GF/B filters soaked with ice-cold PBS. The filter-bound radioactivity was measured as described above. Three to four experiments per compound were performed with each concentration in triplicate.

*β-arrestin 2 recruitment assays*: The measurement of β-arrestin recruitment was performed using the PathHunter assay purchased from DiscoverX (DiscoverX, Birmingham, UK) according to the manufacturer's protocol. HEK293 cells stably expressing β-gal enzyme acceptor were transiently transfected with the ProLink tagged wild-type CXCR3 receptor using the TransIT-293 transfection reagent from Mirus (purchased from MoBiTec, Göttingen, Germany). The assay was carried out in 384-well plates containing 2000 cells in 20 μL. After incubation at 37 °C (5% $CO_2$, 95% relative humidity) overnight, 100 nM CXCL11 and various concentrations of test compounds in PBS, pH 7.4 and 0.20% BSA were added and the incubation continued for 4 h. Detection mix was then added and incubated for a further 60 min at ambient temperature. Chemiluminescence was determined with a microplate reader Victor 3V (Perkin-Elmer, Rodgau, Germany).

*Data analysis*: Data were analyzed by nonlinear regressions using the algorithms in Prism 5.0 (GraphPad Software, San Diego, CA, USA). The equations and models used are in detail described below. Obtained parameters were compared by two-way unpaired *t*-test to estimate the significance in observed differences between the p$K_B$ and $\alpha$ values.

*Binding studies*: The data from the allosteric radioligand displacement assays were analyzed by two different approaches. The data obtained in the homologous competition assays were analyzed by nonlinear regression:

$$Y = \frac{Bottom + (Top - Bottom)}{[1 + 10^{((\log IC_{50} - X) \times HillSlope)}]} \quad (1)$$

Because we often observed incomplete displacement of the radioligand by our novel allosteric ligands, we applied the ternary complex model of allosterism to analyze these data. The data from allosteric radioligand binding studies were fitted to a equation[27] using Prism 5.0:

$$K_{app} = \frac{K_A\left(1 + \frac{[B]}{K_B}\right)}{\left(1 + \frac{\alpha[B]}{K_B}\right)} \quad (2)$$

$$Y = \frac{Y_0\,(1 + K_A)}{([c] + K_{app})} \quad (3)$$

in which $K_{app}$ describes the occupancy of the orthosteric site, $K_A$ is the $K_D$ value of RAMX3 for the investigated receptor, [*c*] the radioligand concentration, [*B*] the concentration of novel allosteric modulator, $K_B$ the equilibrium dissociation constant of modulator binding, and $\alpha$ the ternary complex constant, which denotes the cooperativity factor.[27] The $K_A$ value for the CXCR3 wild-type was set at 14 nM. The concentration of the allosteric radioligand RAMX3 was set to 1 nM. Values of $\alpha > 1$ denote positive cooperativity, whereas $\alpha < 1$ denotes negative cooperativity. Values of $\alpha$ approaching zero are indistinguishable from competitive antagonism. When $\alpha$ approaches zero, the $K_b$ value approaches the $K_i$ value[27].

*Functional studies*: To obtain the $EC_{50}$ values for CXCL11 in wild-type CXCR3 and its mutants, dose–response curves were fitted by nonlinear regression:

$$Y = \frac{Bottom + (Top - Bottom)}{[1 + 10^{((\log IC_{50} - X) \times HillSlope)}]} \quad (4)$$

To characterize the allosteric profile of novel ligands, we applied the ternary complex model of allosterism to analyze the data obtained from the functional assays as described before.[32] The assumptions were that the allosteric modulators do not cause the depression of maximal response or the suppression of the basal activity. The depression of maximal response or the suppression of the basal activity are not accounted for in an allosteric ternary complex model (ATCM). Importantly, even if these assumptions do not hold entirely true for all the novel allosteric modulators, this analysis enables an initial approximation and a semi-empirical estimate of cooperativity. The data from functional studies in which discrete concentrations of agonist CXCL11 and CXCL10 were used, were fitted to following equations using Prism 5.0:

$$K_{app} = \frac{K_A\left(1 + \frac{[B]}{K_B}\right)}{\left(1 + \frac{\alpha\beta[B]}{K_B}\right)} \quad (5)$$

$$Y = \frac{Y_0\,(1 + K_A)}{([c] + K_{app})} \quad (6)$$

in which $K_{app}$ describes the occupancy of the orthosteric site, $K_A$ is the $EC_{50}$ value of CXCL11 or CXCL10 for the investigated receptor, [*c*] the concentration of CXCL11 or CXCL10 used, [*B*] the concentration of novel allosteric modulator, $K_B$ the equilibrium dissociation constant of modulator binding, and $\alpha\beta$ the ternary complex constant, which denotes the cooperativity factor.[27] In the [$^{35}$S]GTP$\gamma$S accumulation assay the $K_A$ value of CXCL11 was set at 1.5 nM and for CXCL10 at 3.3 nM. The concentration of CXCL11 was set to 5 nM and of CXCL10 to 50 nM. In the β-arrestin 2 assay the $K_A$ value CXCL11 was set at 41 nM and for CXCL10 at 7.3 nM. The concentration of CXCL11 was set to 100 nM and of CXCL10 at 40 nM.

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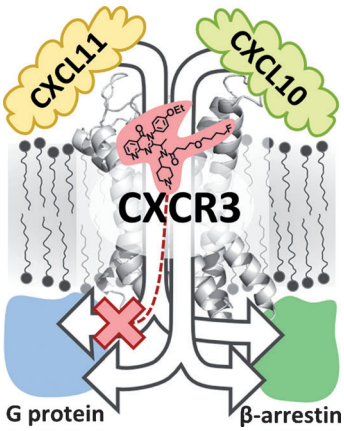
**Ligand-Biased and Probe-Dependent Modulation of Chemokine Receptor CXCR3 Signaling by Negative Allosteric Modulators**




**How amazing can 1 b?** The chemokine receptor CXCR3 is an outstanding platform to study various aspects of allosteric modulation. Herein we report the discovery of a small molecule (compound **1 b**) that can inhibit CXCL11-dependent G protein activation over β-arrestin recruitment with 187-fold selectivity. Compound **1 b** also demonstrates probe-dependent activity: it inhibits CXCL11- over CXCL10-mediated G protein activation with 12-fold selectivity.