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Title:

Molecular mechanisms of biased and probe-dependent signaling at CXCR3 induced by negative allosteric modulators

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Running Title Page

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Abbreviations:

AMs: Allosteric modulators

BRET: Bioluminescence resonance energy transfer

cAMP: Cyclic adenosine monophosphate

cDNA: complementary deoxyribonucleic acid

CXCL9-11: CXC-motif chemokine 9-11

CXCR3: CXC-motif chemokine receptor 3

ELISA: Enzyme-linked immunosorbent assay

GPCR: G protein-coupled receptor

INF-γ: Interferon-γ

MD: Molecular dynamics

OPLS: Optimized Potentials for Liquid Simulations

PCR: Polymerase chain reaction

pK_b: Equilibrium dissociation constant

Rluc: *Renilla* luciferase

Th1: T helper cells

TM: Transmembrane

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α: cooperativity factor

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Abstract

Our recent explorations of allosteric modulators (AMs) with improved properties resulted in the identification of two biased negative AMs, *N*-1-{[3-(4-Ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimi-din2yl]ethyl}-4-(4-fluorobutoxy)-*N*-[(1-methylpiperidin-4-yl)me-thyl}]butanamide (BD103) and {5-[(*N*-{1-[3-(4-ethoxyphenyl)-4-oxo-3,4-dihydropyrido[2,3-d]pyrimidin-2-yl]ethyl-2-[4-fluoro-3-(trifluoromethyl)phenyl]acetamido)methyl]-2-fluorophenyl}boronic acid (BD064), that exhibited probe-dependent inhibition of the chemokine receptor CXCR3 signaling. With the intention to elucidate the structural mechanisms underlying their selectivity and probe-dependency, we used site-directed mutagenesis combined with homology modeling and docking to identify amino acids of CXCR3 that contribute to modulator binding, signaling and transmission of cooperativity. With the use of allosteric radioligand RAMX3 we identified F131$^{3.32}$ and Y308$^{7.43}$ that contribute specifically to the binding pocket of BD064, whereas D186$^{4.60}$ solely participate in the stabilization of binding conformation of BD103. The influence of mutations on the ability of negative allosteric modulators to inhibit the chemokine-mediated activation (CXCL11 and CXCL10) was assessed with BRET based cAMP and β-arrestins recruitment assay. Obtained data revealed complex molecular mechanisms governing biased and probe-dependent signaling at CXCR3. In particular, F131$^{3.32}$, S304$^{7.39}$ and Y308$^{7.43}$ emerged as key residues for the compounds to modulate the chemokine response. Notably, D186$^{4.60}$, W268$^{6.48}$ and S304$^{7.39}$ turned out to play a role in signal pathway selectivity of CXCL10 as mutations of these residues led to a G protein active but β-arrestin inactive conformation. These diverse effects of mutations suggest the existence of ligand- and pathway-specific receptor conformations and give new insights in the sophisticated signaling machinery between allosteric ligands, chemokines and their receptors, which can provide a powerful platform for the development of new allosteric drugs with improved pharmacological properties.

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Introduction

Biased allosteric modulation of G protein-coupled receptors represents an attractive approach to drug design (Khoury et al., 2014; Leach et al., 2007c). While allosteric ligands act at sites that are topographically distinct from the orthosteric binding site, they display unique properties that are reflected in greater receptor subtype selectivity, permissivity, saturability of effect, probe-dependence and biased signaling (Christopoulos, 2002; Kenakin, 2010, 2012). The latter has attracted special attention in the past decade as some ligands have emerged that stabilize diverse active receptor states, which resulted in a selective biological response (Kenakin, 2007). We recently discovered biased allosteric agonists of the chemokine receptor CXCR3 that either lead to β-arrestin 2 recruitment and internalization or solely activate G proteins (Milanos et al., 2016a). Another interesting phenomenon with substantial implications for drug discovery is related to the permissive nature of allosteric agents and is known as probe-dependence. In this case, the allosteric effects can vary depending on the co-bound orthosteric ligand ("probe") and are manifested by a change in cooperativity (Kenakin, 2005; Leach et al., 2007b). For example, the well-characterized allosteric anti-HIV modulator of the chemokine CCR5 receptor, aplaviroc, can selectively block the binding of [^{125}I]-MIP1α (CCL3) while the binding of [^{125}I]-RANTES is unaffected (Watson et al., 2005).

The chemokine system itself is an excellent example for studying functional selectivity. The promiscuity of chemokines with multiple shared ligands and receptors is often wrongly referred to as redundancy. However, several studies have shown that the binding of different chemokines to a single chemokine receptor can differ in the biological response resulting in functional selectivity (Corbisier et al., 2015; Leach et al., 2007a; Rajagopal et al., 2013; Tian et al., 2004). This is also the case for the chemokine receptor CXCR3, which is activated by gamma-inducible chemokines CXCL11, CXCL10 and CXCL9 (Groom and Luster, 2011). Dysfunctions of the CXCR3 signaling network are linked to a myriad of inflammatory diseases, such as rheumatoid arthritis (Lacotte et al., 2009), multiple sclerosis (Sorensen

et al., 1999), cancer (Kawada et al., 2007; Ma et al., 2009), atherosclerosis (Mach et al., 1999) and allograft rejection (Cox et al., 2001). Therefore, intensive efforts have been devoted to developing allosteric modulators as therapeutics with greater potential for treating such diseases through enhanced selectivity (Bernat et al., 2014; Scholten et al., 2012; Wijtmans et al., 2008). Despite the strong focus in small-molecule ligands for CXCR3, the lack of structural data on chemokine binding and activation makes it challenging to understand the precise mechanisms of the interactions of allosteric modulators with the receptors. Nonetheless, some crystal structures of chemokine receptors including CXCR4 in complex with small-molecule antagonists IT1t and CVX15 (Wu et al., 2010) or the viral chemokine vMIP-II (Qin et al., 2015), afforded some structural insights in the interaction between ligands. Three GPCR binding pockets have been identified; 1T1t was detected to interact with residues in the minor binding pocket, CVX15 with residues in the major pocket and vMIP-II extending to both subpockets (Nicholls et al., 2008; Salchow et al., 2010). Site-directed mutagenesis has also been used to dissect the binding mode of novel allosteric modulators of CXCR3. Scholten et al. showed that NBI-74330 is mainly anchored into the minor pocket, whereas VUF11211 extends from the minor pocket in the major pocket (Scholten et al., 2014). In addition, Nedjai et al. described the binding site of the CXCR3 antagonist VUF10085 in the minor pocket with Asp112$^{2.63}$, F131$^{3.32}$ and Y308$^{7.43}$ forming direct contact points, but distinct from the broad-spectrum antagonist TAK-779, which is assumed to bind outside the minor pocket (Nedjai et al., 2015).

Our recent explorations of allosteric modulators with improved properties resulted in the identification of two biased negative allosteric modulators that exhibited probe-dependent inhibition of CXCR3 signaling (Bernat et al., 2015). Such probe-dependent allostery may serve to fine tune the chemokine response in the seemingly redundant area of multiple chemokine agonists for receptors. In the current study, we aimed to elucidate the binding mode of our novel allosteric modulators and to identify amino-acid residues that steer the bias and probe-dependent mechanisms using site-directed mutagenesis and

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computational studies. By doing so, we provide novel insights into the complex interactions between allosteric ligands, chemokines and their receptor, which open a powerful platform for further development and rational design of allosteric modulators with improved bias and probe-dependency. Moreover, we report for the first-time amino-acid residues that are specifically involved in CXCL10-mediated β-arrestin recruitment but not in G protein activation.

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Materials and Methods

CXCR3 Ligands: The synthesis of cRAMX3 was previously described (Bernat et al., 2014). BD064 [compound 14 in the study by Bernat et al. (2014) (Bernat et al., 2014)] and BD103 [compound 1b in the study by Bernat et al. (2015) (Bernat et al., 2015)] were synthesized according to the previously described procedures (Bernat et al., 2014; Bernat et al., 2015). Briefly, for the synthesis of the boronic acid derivative BD064 the primary amine (2-(1-aminoethyl)-3-(4-ethoxyphenyl)pyrido[2,3-d]pyrimidin-4(3H)-one) was reductively alkylated using the protocol for reductive amination developed by Molander et al. (Molander and Cooper, 2008). The required trifluoroborate-substituted aromatic aldehyde was prepared from commercially available boronic acid ((2-flouro-5-formylphenyl)boronic acid) by treatment with potassium hydrogen difluoride in methanol. The resulting secondary amine was subsequently acylated by activated 2-[4-fluoro-(3-trifluoromethyl)phenyl]acetic acid. The Trifluoroborate moiety was converted to boronic acid by the treatment with trimethylsilyl chloride in aqueous acetonitrile

The synthesis of BD103 was similar. Also the primary amine (2-(1-aminoethyl)-3-(4-ethoxyphenyl)pyrido[2,3-d]pyrimidin-4(3H)-one) was reductively alkylated using corresponding substituted benzaldehyde and triacetroxyborohydride. The resulting secondary amine was subsequently acylated by 4-(4-fluorobutoxy)butanoic acid, which was synthesized according to the previously published procedure (Bernat et al., 2014).

Modeling and docking of BD064 and BD103: The human CXCR3 homology model used in our docking studies was that reported previously (Milanos et al., 2016b). Ligand structures were optimized using OPLS force field (Harder et al., 2016) in the LigPrep tools and the relevant protonation states at physiological pH were determined using Epik. The ligands were docked using a 25-Å box centered on cRAMX3 and default settings in GLIDE standard precision (SP) (Halgren et al., 2004).

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Cell Culture and Transfection: Human embryonic kidney (HEK) 293 T cells were grown at 37°C and 5% CO_2 in DMEM/F-12 (Gibco, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin-streptomycin. Cells were transiently transfected with 5 µg of pcDNA 3.1 containing either FLAG-CXCR3 wild type or mutants using TransIT-293 transfection reagent (Mirus Bio, Madison, WI, USA)). Cells were harvested after 48 h for membrane preparations. For the cAMP biosensor assay, HEK293T cells were transiently transfected with 1 µg/ml of wild type or mutant CXCR3 and 2 µg/ml of DNA encoding for the cAMP biosensor CAMYEL (cAMP sensor using YFP-Epac-RLuc, American Type Celture Collection, Manassas, VA, USA) (Jiang et al., 2007). For β-arrestin recruitment experiments, HEK293T cells were transfected with cDNA coding for β-arrestin1- or 2-*R*luc and CXCR3-mVenus (either wild type or mutant) and linear polyethyleneimine (linear PEI, 25 kDA, Polysciences Inc., Warrington, PA, USA) in a 1:3 ratio. Cells were plated in 96-well F-Bottom white tissue culture plates (Greiner-Bio-One GmbH, Frickenhausen, Germany) at a density of 3×10^5.

DNA Constructs and Site-Directed Mutagenesis: The wild type CXCR3 was a kind gift of Prof. Dr. R. Leurs (VU Amsterdam, Netherlands). cDNA encoding for the BRET based cyclic AMP (cAMP) was purchased from American Type Culture Collection (Manassas, VA, USA) (pcDNA3.1-(L)-His-CAMYEL #ATCC-MBA-277). The pcDNA3.1-β-arrestin1/2-*Renilla* luciferase (Rluc) constructs were kind gifts from Dr. M. Bouvier (University of Montréal, QC, Canada). The FLAG-CXCR3-mVenus construct was generated by substituting the stop codon of CXCR3 and fusing them to yellow fluorescent protein (mVenus). We generated the point mutations by polymerase chain reaction using oligonucleotide primers with desired amino acid change and either pc DNA 3.1 CXCR3 with a FLAG tag encoded at the N terminus or pc DNA 3.1 FLAG-CXCR3 with a C-terminal mVenus as a template. Amplification conditions were 10 s at 98 °C, 15 s at 68 °C and 30 s at 72 °C for 30 cycles. The PCR amplified fragment was digested with BmtI and XhoI. All mutations were verified by restriction endonuclease mapping and DNA sequence analysis (LGC Genomics, Berlin, Germany).

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Membrane Preparation: At 100% confluency cells were washed with PBS twice, 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, resuspended in Tris-EDTA-$MgCl_2$ buffer (50mM 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. After centrifugation at 50,000×g, at 4 °C for 18 min the membranes were resuspended in the 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 with the Lowry method with bovine serum albumin used as a standard (Lowry et al., 1951).

Enzyme-Linked Immunosorbent Expression Assay (ELISA): The day after transfection, cells were detached by trypsin, resuspended into fresh culture medium and plated in poly(D-lysine)-coated 48-well assay plates. After additional 24h the cells were fixed with 4% formaldehyde solution (Roti®-Histofix 4%, Carl Roth GmbH, Karlsruhe, Germany). After three washes with washing buffer (150 mM NaCl, 25 mM Tris, pH 7.5) and being blocked with 3% skim milk in washing buffer pH 8, cells were incubated with monoclonal mouse ANTI-FLAG® M2 antibody (Sigma Aldrich, Steinheim, Germany) in the blocking buffer. After 1 h the cells were washed three times and incubated with Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit (Sigma Aldrich). Subsequently, cells were incubated with substrate buffer containing 2 mM *o*-phenylenediamine, 35 mM citric acid, 66 mM Na_2HPO4 and 0.015% H_2O_2 at pH 5. The coloring reaction was stopped with 1 M H_2SO_4 and the absorption at 490 nm was determined using a CLARIOstar® microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

Allosteric Radioligand Displacement Assay: Receptor binding studies were performed on membrane preparations of HEK293T cells expressing the corresponding receptor. The enantiopure tritium labeled RAMX3 (specific activity: 85.8 Ci/mmol) at the concentration of 1 nM was used for the assays. To determine unspecific binding 5μM of cRAMX3 was used. The assays were carried out in 96 well plates

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at a protein concentration of 30 µg/mL 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 at 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, Germany). The filters were rinsed five times with ice cold Tris-NaCl buffer. After drying for 3h at 60 °C, filters were sealed with melt-on scintillator sheets Melti-Lex G/HS (Whatman/Perkin-Elmer, Freiburg, Germany) and the trapped radioactivity was measured in a microplate scintillation counter (Micro Beta Trilux scintillator, Whatman/Perkin-Elmer). Three to five experiments per compound were performed with each concentration in triplicate.

cAMP Biosensor Assay: The experimental procedure for this assay has been adapted as previously described (Barak et al., 2008). HEK293T cells transiently transfected with the CAMYEL sensor and the corresponding receptor were split into 96-well half area white tissue culture plates (Greiner-Bio-One GmbH, Frickenhausen, Germany) at a density of 2×10^4 cells per well. The next day, cells were incubated with 30 µl Hank`s balanced salt solution (HBSS) for 1 h before treatment. Next, 10 µl substrate coelenterazine-h (Promega GmbH, Mannheim, Germany) was added to a final concentration of 5 µM. To measure the effects of ligands on cAMP levels, chemokine alone or EC_{80} of chemokine and various concentration of negative allosteric modulator in HBSS buffer containing 50 µM forskolin (Sigma Aldrich) and 0.2% BSA were added 5 min after coelenterazine-h. After additional 15 min of incubation the YFP emission (535-30 nm), as well as the *R*luc emission (475-30 nm) were measured using a CLARIOstar® microplate reader. The BRET signal (BRET ratio) was determined by calculating the ratio between the YFP and the *R*luc emission.

β-Arrestin BRET Assay: Cell plating and transfection was performed as mentioned earlier. Forty-eight hours post-transfection, the medium was exchanged with 80 µl HBSS and incubated 30 min before treatment. The coelenterazine-h was added at a final concentration of 5 µM. After 5 min. of incubation, chemokine alone or EC_{80} of chemokine and various concentration of negative allosteric modulators were

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added and incubated for an additional 10 min. The plate was measured on a CLARIOstar® microplate reader and BRET ratios were calculated.

Data Analysis: The data was analyzed by nonlinear regressions using the algorithms in PRISM 6.0 (GraphPad Software, La Jolla, CA, USA). The equations and models used are described in detail below. Obtained parameters were compared by one-way ANOVA followed by Dunnett`s Multiple Comparison Test to estimate the significance in observed differences between wild type CXCR3 and its mutants. The data from allosteric radioligand displacement assays were analyzed by two different approaches. The data obtained in the homologous competition assays were analyzed by nonlinear regression:

$$Y= \frac{\text{bottom} + (\text{top-bottom})}{\left[1+10^{(\log EC_{50}-X)\cdot \text{Hillslope})}\right]} \quad (1)$$

Because we often observed incomplete displacement of the radioligand by our negative allosteric modulators, we applied the ternary complex model of allosterism to analyze these data. The data from allosteric radioligand binding studies were fitted to an equation (Christopoulos and Kenakin, 2002) using PRISM 6.0:

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

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

where K_{app} describes the occupancy of the orthosteric site, K_A is the K_D value of RAMX3 for the investigated receptor, [c] is the concentration of the radioligand, [B] is the concentration of allosteric modulator, K_B is the equilibrium dissociation constant of modulator binding, and α is the ternary complex constant, which denotes cooperativity factor (Christopoulos and Kenakin, 2002). Values of $\alpha > 1$ denote positive cooperativity, whereas $\alpha < 1$ denotes negative cooperativity. Values of α approaching zero are indistinguishable from competitive antagonism. When α approaches zero, the K_b value approaches the K_i value (Christopoulos and Kenakin, 2002). An α value equal to 1 denotes an allosteric interaction that result in an unaltered ligand affinity.

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To obtain the EC_{50} and E_{max} (maximal response) values for CXCL11 and CXCL10 in wild type CXCR3 and its mutants from cAMP BRET and β-arrestin BRET Assays, dose-response curves were fitted by nonlinear regression and normalized to the CXCR3 WT response:

$$Y = \frac{\text{bottom} + (\text{top-bottom})}{\left[1+10^{(\log EC_{50}-X)\cdot \text{Hillslope})}\right]} \quad (4)$$

Subsequently, the index of agonism $\log(max/EC_{50})$ was calculated to quantify the effects of receptor mutation on CXCL11- and CXCL10-mediated signaling. The parameter is derived from agonists concentration-response curves and comprises the maximal response to the agonist (max) and the EC_{50}. Finally, comparisons of $\log(max/EC_{50})$ values between mutants yielded $\Delta\log(max/EC_{50})$, which allows the system independent scaling of agonism within a given functional system. When this is done, the agonism of the agonist at mutant CXCR3 is compared with the agonist at WT CXCR3 and system effects are cancelled (Kenakin, 2017).

$$\Delta\log(max/EC_{50}) = \log(max/EC_{50})_{WT} - \log(max/EC_{50})_{mutant} \quad (5)$$

To account error propagation, standard errors of the mean for the normalized $\Delta\log(max/EC_{50})$ values were calculated employing equation 6.

$$S.E.M._{1,2} = \sqrt{(S.E.M._1)^2 + (S.E.M._2)^2} \quad (6)$$

To account for the change in activity between the two chemokines CXCL11 and CXCL10 the $\Delta\Delta\log(max/EC_{50})$ values were calculated for each mutant. For WT CXCR3 the $\Delta\Delta\log(max/EC_{50})$ was set to 0.

$$\Delta\Delta\log(max/EC_{50}) = \Delta\log(max/EC_{50})_{CXCL10} - \Delta\log(max/EC_{50})_{CXCL11} \quad (7)$$

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Results

We previously described a series of novel negative allosteric modulators with biased functional properties (Fig. 1). BD064 preferentially inhibits CXCL11-mediated β-arrestin 2 recruitment over G protein activation, whereas BD103 preferably blocks CXCL11-mediated activation of G proteins rather than β-arrestin 2 recruitment (Bernat et al., 2015).

Based on the homology model of CXCR3 and docking studies, our aim was to define the binding mode of given biased negative allosteric modulators experimentally and to dissect the molecular mechanisms responsible for biased signaling. We therefore combined site-directed mutagenesis with detailed functional characterization of mutant receptors in allosteric radioligand displacement and BRET-based cAMP and β-arrestin recruitment assays.

Computational studies on the binding mode of BD064 and BD103. We recently determined a binding mode for the RAMX3, a closely related CXCR3 antagonist, to its CXCR3 receptor based on a homology model after 1.8 μs of molecular dynamics simulation using metadynamics enhanced sampling (Milanos et al., 2016b). Two closely related biased negative allosteric agonists BD064 and BD103 were then docked based on this binding mode. The binding mode of BD064 is analogous to that of cRAMX3 (Milanos et al., 2016b). BD064 spans from the minor to the major pocket, mainly binding to the interface between these two pockets and interacting with residues in TM3, 6 and 7 of CXCR3 (Fig. 2). Specifically, F131$^{3.32}$ can form π-π stacking interactions with both the aromatic bicyclic unit and the boron phenyl ring of BD064. Additional π-π stacking interactions are observed with Y271$^{6.51}$ and Y308$^{7.43}$. K300$^{7.35}$ hydrogen bonds to the nitrogen atom of the heterocycle. Further amino acids that were not tested by mutagenesis appear to interact with BD064 in the proposed binding pocket including F135$^{3.36}$, W109$^{2.60}$, H272$^{6.52}$ and D278$^{6.58}$. The binding mode of BD103 differed from those of cRAMX3 and BD064. It sits higher and further left towards TM 5and 6 in the binding cavity of CXCR3 (Fig. 3). The binding site is

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mainly defined by residues from TM 5, 6 and 7. The upward shift of the binding pocket of BD103 is predominantly caused by the cation-π interaction of the piperidine moiety with $Y205^{ECL2}$, which prevents BD103 binding to the deep binding pocket of cRAMX3 and BD064. Two additional residues contribute to this "hot spot"; $R216^{5.39}$ and $D186^{4.60}$ build an ionic lock to stabilize the binding conformation of BD103. $F131^{3.32}$ and $Y308^{7.43}$, important anchor residues for BD064 and cRAMX3, do not interact with BD103 due to the higher binding site and the resulting increased distance between the modulator and these two residues. The binding pocket of BD103 partially overlapped with that of BD064 and shared the residues $Y271^{6.51}$ and $W109^{2.60}$, which are predicted to form multiple π- π stacking interactions with BD103. Additionally, the methoxyphenyl moiety of BD103 interacted with $I279^{6.59}$, $V275^{6.55}$, $T213^{5.36}$ and $V217^{5.40}$ via lipophilic interactions.

Influence of amino acid substitutions on the affinity and cooperativity of biased negative allosteric modulators towards RAMX3. Changes in the receptor sequence induced by site-directed mutagenesis can have significant impact on the receptor expression level. The expression of the CXCR3 receptor mutants was analyzed by whole cell-based ELISA. HEK293T cells were transiently transfected with cDNA coding for FLAG-CXCR3 wild type or mutant receptors. The cell surface expression level of mutants was found to be between 61% and 109% of the wild type expression (Supplemental Information Table S1). The mutations $W268^{6.48}$F and$Y271^{6.51}$F resulted in a most significant reduction of the receptor surface expression, reaching only about 60% of the wild type expression.

To examine the effects of amino-acid substitutions on the binding of the allosteric modulators, we next performed an allosteric radioligand (RAMX3) (Bernat et al., 2012) displacement assay. The use of allosteric radioligand enables the measurement of affinity of the allosteric ligands directly for their allosteric binding pocket in a competitive manner. In this assay, the membrane preparations of HEK293T cells that express the corresponding receptor were used. In general, the specific binding detected in this assay correlated well with the ELISA findings (Supplemental Information Fig. S1, Table S1). Decreased

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level of bound RAMX3 at the W268$^{6.48}$F mutant correlates with a reduced receptor expression as determined by ELISA. The mutation K300$^{7.35}$R with an expression level comparable to the wild type, resulted in strongly reduced radioligand binding, suggesting that this residue is an important anchor for the binding of RAMX3 to CXCR3. Remarkably, reduced surface expression level of the Y271$^{6.51}$F mutant did not influence the extent of specific RAMX3 binding. Most likely, this mutation influences the extent of the receptor cell surface expression, but not the overall expression of the protein. Membrane preparations were used for the radioligand binding-studies that include not only the plasma membrane but also other intracellular membranes that might contain immature receptors. The ELISA assay only detects mature receptors expressed on the surface.

Next, the ability of cold RAMX3 (cRAMX3) and two biased negative allosteric modulators BD064 and BD103 to displace RAMX3 from the wild type and mutant receptors was evaluated. We previously showed that BD064 and BD103 were unable to suppress the binding of RAMX3 to CXCR3 completely, so that the ternary complex model of allosterism was used to analyze the radioligand binding data (Bernat et al., 2014; Bernat et al., 2015). This model was also used here to analyze the radioligand binding data. It describes allosteric interactions only in terms of the equilibrium dissociation constant pK_b and the cooperativity factor α. Values of $\alpha > 1$ denote positive and $\alpha < 1$ negative cooperativity. Values of α approaching zero are indistinguishable from competitive antagonism. $\alpha = 1$ denotes an allosteric interaction that results in unaltered ligand affinity (Christopoulos and Kenakin, 2002; Ehlert, 1988). The pK_b and α values from these experiments are listed in Supplemental Information Table S1 and depicted on Fig. 4; none of the mutants had a significant effect on the affinity of cRAMX3. As expected, the binding cooperativity between the allosteric radioligand RAMX3 and cRAMX3 was indistinguishable from competitive antagonism with α equaling or approaching zero. As reported previously, BD064 displayed nearly the same affinity as cRAMX3 at the wild type but weaker negative cooperativity against RAMX3 with α value of 0.38 ± 0.05 (Bernat et al., 2014; Bernat et al., 2015). Consistent with our

hypothesis that cRAMX3 and BD064 can bind to CXCR in different orientations (Bernat et al., 2014; Bernat et al., 2015), few mutations influenced the BD064 affinity. Specifically, alanine substitution of the residue Y308$^{7.43}$ led to a 15-fold loss in affinity of BD064. An even more drastic effect was observed at the F131$^{3.32}$A and S304$^{7.39}$E mutant, where the low binding affinity did not allow pK_b and α values to be calculated. These results indicate that Y308, F131 and S304 contribute substantially to the binding of BD064 to CXCR3, but not RAMX3. The negative cooperativity between BD064 and RAMX3 was significantly reduced only at the W268$^{6.48}$F mutant (α value of 0.38 vs. 0.72), thus suggesting that this residue plays a role in the transmission of cooperativity between BD064 and the radioligand RAMX3 and does not contributes to the binding affinity. For BD103 the observed affinity for wild type CXCR3 was a somewhat lower than previously reported with a comparable incomplete displacement of the radioligand (α value of 0.22) (Bernat et al., 2015). The S304$^{7.39}$E mutant, which displayed a large reduction in affinity for BD064, also caused a striking loss of affinity for BD103. A considerable effect on the binding of BD103 was also observed for the mutation of D186$^{4.60}$N. In contrast to BD064 the mutation F131$^{3.32}$A had no effect on affinity and cooperativity of BD103. The residues S304$^{7.39}$ and D186$^{4.60}$ are thus essential for the binding of BD103 to CXCR3. The serine mutation S301$^{7.36}$A, which did not have any significant influence on binding affinity of BD103, resulted in a significant weakening of the negative cooperativity between BD103 and RAMX3 (α value of 0.47 vs. 0.22). All other mutants had no appreciable effect on the affinity and cooperativity of BD103 towards RAMX3.

In summary, the radioligand displacement experiments confirmed that the biased allosteric modulators BD064 and BD103 bind to overlapping allosteric sites in CXCR3, and thus react to mutations differently. Key residues like F131$^{3.32}$, D186$^{4.60}$ and Y308$^{7.43}$ arose as the most important anchor points for the investigated biased negative allosteric modulators. These interactions are also consistent with the proposed binding mode from the docking studies.

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Effects of Mutations on Signaling of Chemokines CXCL11 and CXCL10. Before investigating the interplay between the endogenous agonists and biased negative allosteric modulators, we investigated the influence of mutations on chemokine signaling in G protein-dependent and independent pathways. CXCR3 is a Gαi-coupled receptor, thus the G protein-dependent signaling can be monitored as a change in the intracellular cAMP level (Denis et al., 2012). While in the G protein-independent signaling β-arrestin 1 and β-arrestin 2 are involved (Oakley et al., 2000), which evoke isomer-specific functions (Ahn et al., 2004; Kohout et al., 2001), we decided to monitor the influence of mutations on both pathways. We chose BRET-based approaches for all three functional assays. BRET allows the real-time detection of protein-protein interactions in living cells (Eidne et al., 2002). For the quantitatively and rapid monitoring of intracellular levels of cAMP we used the Epac based BRET sensor CAMYEL (Jiang et al., 2007). CXCR3 couples to $G\alpha_i$, which leads to a negative regulation of the adenylate cyclase and a decrease in cAMP levels (Denis et al., 2012) and requires a pre-stimulation of adenylate cyclase with forskolin. To measure the recruitment of β-arrestin with BRET, CXCR3 was fused to the modified EYFP (mVenus) and β-arrestin was fused to Rluc as described by Hamdan *et al*. (Hamdan et al., 2005). Agonist concentration-response curves were fitted by nonlinear regression to yield Emax (maximal response) and EC_{50} (Supplemental Information Table S2-S4) and subsequently analyzed applying the $\Delta\log(max/EC_{50})$ scale (Fig. 5). Subsequently the $\Delta\Delta\log(max/EC_{50})$ values were calculated for the investigated pathways to get the change in CXCL10 activity vs. CXCL11 at each mutant (Fig. 6). This single index of agonism allows a system-independent comparison of agonist potencies and signaling efficacies across different receptor mutants in that the effects of the system processing of agonist response and differences in assay sensitivity and receptor expression are cancelled (Kenakin, 2017).

All mutant receptors were readily activated by CXCL11 and CXCL10 as shown in a dose-dependent inhibition of forskolin-stimulated cAMP production. However, some mutations induced the opposite effect on the activation of G proteins by either decreasing or increasing the signaling depending on the

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chemokine used. W268$^{3.32}$F and D297$^{7.32}$N gave a five to eight-fold reduction in the potency of CXCL11 and CXCL10, although for CXCL11 the change in log(max/EC_{50}) was not statistically significant (Fig. 5 and 6). However, the efficacy of CXCL11 increased significantly at these mutations, but not that of CXCL10. The mutation D186$^{4.60}$N resulted in a drastic increase of CXCL11 efficacy (+51%) with no influence on potency. This mutation led, on the other hand, to the most dramatic drop in potency of CXCL10 and therefore to a nearly 10-fold decrease in relative activity of CXCL10 over CXCL11 (Fig 6). In contrast, the mutation Y271$^{6.51}$F produced a 2-fold selective decrease on the activity of CXCL11 over CXCL10 (Fig. 6).

The influence of mutations on the CXCL11- and CXCL10- mediated recruitment of β-arrestins differed significantly from their influence on the G protein mediated signaling (Supplemental Information Table S3 and S4, Fig. 5 and 6). Mutation of D297$^{7.32}$ to asparagine led to reduced efficacy of both chemokines (31% and 49%, respectively) in recruiting β-arrestin 1, without affecting β-arrestin 2 recruitment (Table 3 and 4). Moreover, this mutant had no influence on the potency of CXCL11, but induced a 16-fold reduction in recruitment for β-arrestin 1 and 7-fold for β-arrestin 2 (Fig. 7A and B). Similarly, exchange of S301$^{7.36}$ for alanine had no effect on the CXCL11- and CXCL10-mediated β-arrestin 2 recruitment, yet both the efficacy of CXCL11 and CXCL10 and the potency were decreased for β-arrestin 1 recruitment (Supplemental Information Table S3 and S4, Fig. 5). Both alanine mutations of F131$^{3.32}$ and Y308$^{7.43}$ led to a pronounced decrease in CXCL11 mediated recruitment of β-arrestins (Fig. 5 and 6). The substitution of K300$^{7.35}$ by either methionine or arginine did not affect the recruitment of β-arrestin mediated by either CXCL11 or CXCL10. The mutations D186$^{4.60}$N, W268$^{6.48}$F and S304$^{7.39}$E almost completely abolished the CXCL10-mediated β-arrestin 1 and 2 recruitment to CXCR3. It is important to note, that this effect was exclusively observed for CXCL10 (Supplemental Information Table S3 and S4, Fig 6). However, CXCL11 was still able to mediate the recruitment of β-arrestin to D186$^{4.60}$N and W268$^{6.48}$F mutants, despite a significant loss of efficacy. The mutation S304$^{7.39}$E had no effect at all on

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CXCL11- mediated β-arrestin-recruitment. Although the mutation S304$^{7.39}$E almost completely abolished the CXCL10-mediated recruitment of β-arrestins to CXCR3 wild type, this mutation had no influence on CXCL10- mediated G protein signaling (Fig. 5). Collectively, D186$^{4.60}$, W268$^{6.48}$ and S304$^{7.39}$ play a role in signal pathway selectivity as mutations of these residues led to a G protein active but β-arrestin inactive conformation.

In general, the mutations had a pronounced effect on the potency of CXCL11- and CXCL10- mediated G protein activation, in contrast to their effect on CXCL11- and CXCL10- mediated β-arrestin recruitment, where the efficacy was strongly influenced. These observations are in accord with the accepted notion that different receptor conformations initiate either G protein activation or recruitment of β-arrestin (Liu et al., 2012; Rahmeh et al., 2012). Moreover, these conformations can also differ depending on the bound chemokine, which consequently results in a probe-dependent behavior (Fig. 6).

Effects of Mutations on the Ability of Negative Allosteric Modulators to Inhibit Chemokine - mediated cAMP Signaling. The binding of compounds to the allosteric site on a GPCR changes the receptor conformation and can therefore affect the binding affinity and /or the signaling efficacy of the orthosteric ligand (May et al., 2007). To estimate the influence of mutations on the ability of allosteric modulators to modulate the potency and efficacy of CXCL11 and CXCL10, we first analyzed their influence on the G protein-dependent signaling (Supplemental Information Table S5 and S6). The results were analyzed using a simple allosteric ternary complex model as described before (Bernat et al., 2014). The assumptions were that the allosteric modulators neither depress the maximal response nor suppress the basal activity. These effects are not accounted for in an allosteric ternary complex model. Importantly,

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even if these assumptions are not entirely true for all the allosteric modulators, this analysis enables an initial approximation and a semi-empirical estimate of cooperativity (Christopoulos and Kenakin, 2002).

All three negative allosteric modulators suppressed the chemokine-induced activation at CXCR3 with the following rank order of functional affinities: cRAMX3 > BD064 > BD103, where slight probe dependence was observed for BD064. Compound BD064 inhibited the response of CXCL10 more strongly than that of CXCL11 (6.5-fold difference in pK_b: 6.69 vs. 7.50, two-tail unpaired t-test, $p < 0.05$, Supplemental Information Table S5 and S6), in accord with the results of an [^{35}S]GTPγS incorporation assay (Bernat et al., 2015). The modulators demonstrated behavior undistinguishable from competitive antagonism with α equal or nearly zero. Neutralizing the two aspartic acid residues D186$^{4.60}$ and D297$^{7.32}$ by mutation to asparagine generally improved the functional affinity of cRAMX3 and BD064. A significant improvement was observed for BD064 to modulate CXCL11-mediated G protein activation at the D186$^{4.60}$N mutant (7.7-fold, Fig. 7I) and for cRAMX3 to modulate CXCL10-mediated G protein activation at D186$^{4.60}$N and D297$^{7.32}$N (12-fold and 8-fold, respectively, Fig. 8B). The mutations D186$^{4.60}$N and D297$^{7.32}$N resulted in enhanced negative cooperativity between BD103 and CXCL11. Furthermore, cRAMX3 was more potent at Y271$^{6.51}$F to inhibit CXCL11- and CXCL10- mediated cAMP response (5-fold and 7-fold, respectively). The substitution of K300$^{7.35}$ for methionine resulted in a 6-fold pK_b improvement of cRAMX3 to modulate CXCL10-induced G protein signaling. However, this mutation induced a 5.5fold loss of functional affinity and cooperativity between BD064 and CXCL11 (Fig. 7D). Moreover, this effect was probe-dependent because the mutation K300$^{7.35}$M did not affect BD064 ability to inhibit CXCL10-mediated response. Substitution with arginine at the same position was tolerated for all given negative allosteric modulators as well as the mutation of S301$^{7.36}$ to alanine. The mutation of W268$^{6.48}$ to phenylalanine caused a large reduction (8-fold) in cRAMX3 ability to inhibit CXCL11-mediated activation (Supplemental Information Table S5), but had no effect on BD064 and BD103. The same mutation resulted in a minor reduction (3-fold) of BD064 ability to inhibit CXCL10-

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mediated activation (Fig. 8E) and a complete loss of BD103 activity towards CXCL10 (Fig. 8H). The most striking effects on the signaling properties of the negative allosteric modulators were observed at F131$^{3.32}$A, Y308$^{7.43}$A and S304$^{7.39}$E. Regarding CXCL11-mediated G protein activation, F131$^{3.32}$A let to a considerable lower pK_b of cRAMX3 (27-fold) and BD064 (4.5-fold) and a weaker negative cooperativity between BD064 and CXCL11 (23-fold, Fig. 7A-C). In contrast, this mutation had no significant effect on the ability of both negative allosteric modulators to inhibit CXCL10-mediated activation indicating a probe-dependent effect of this mutant (Fig. 8A and D). Likewise, strong probe-dependence was detected by the exchange of Y308$^{7.43}$ for alanine, which caused a dramatic reduction of the functional affinity and cooperativity of cRAMX3 to inhibit CXCL11-mediated signaling (19.5-fold and 57-fold, respectively, Fig. 7G), whereas only the functional affinity of cRAMX3 towards CXCL10 was decreased (26-fold) with a nearly unchanged cooperativity (α value of 0.07). In addition, this mutation seems to play an even more important role for the functional activity of BD064 as the pK_b and α value could not be determined accurately because of the pronounced reduction in signaling. Although this compound was still able to inhibit CXCL10-mediated G protein activation at the Y308$^{7.43}$ mutant, the cooperativity and functional affinity were substantially decreased (30-fold and 56-fold, respectively). The last mutant S304$^{7.39}$E displayed a significant drop in functional affinity and cooperativity for cRAMX3 and BD064 to inhibit the chemokine elicited response (Fig. 7E). As reported earlier the residues involved in the signaling of BD103 were difficult to detect due to poor affinity, but we noted some slight weaker negative cooperativity for the Y271$^{6.51}$F, K300$^{7.35}$M, S301$^{7.36}$A and Y308$^{7.43}$A (Supplemental Information Table S5 and Fig. 7H) mutant in presence of CXCL11 as well as for the D297$^{7.32}$N, K300$^{7.36}$M, S304$^{7.39}$E and Y308$^{7.43}$A (Supplemental Information Table S6 and Fig. 8I) mutant in the presence of CXCL10. The residues contributing to the signaling transduction of BD103 appear to differ in some parts compared to cRAMX3 and BD064, consistent with the results from the radioligand binding studies. The cAMP BRET assay also showed that at some mutants (F131$^{3.32}$A, D297$^{7.32}$N,

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K300$^{7.35}$R and Y271$^{6.51}$F) the modulators elicited higher levels of cAMP as the basal level pre-stimulated with forskolin (e.g. Fig. 8A). This indicates inverse agonistic activity of the modulators, which was confirmed by experiments without the addition of endogenous ligand (Supplemental Information Fig. S2). In general, the data suggest that the amino-acid residues involved in the binding of the modulators (F131$^{3.32}$A, Y308$^{7.43}$A and S304$^{7.39}$E), also play an important role in signaling transmission. However, there is a need to differentiate the modifications, that influence the compound affinity, from those that influence the cooperativity exhibited toward the endogenous ligand, as the two properties are not correlated (Kenakin and Miller, 2010; Keov et al., 2011).

Effects of Mutations on the Ability of Modulators to Inhibit Chemokine - mediated β-Arrestin Recruitment. To evaluate the importance of amino-acid substitutions for β-arrestin biased negative allosteric modulation, we tested cRAMX3, BD064 and BD103 for their ability to inhibit the chemokine-induced recruitment of β-arrestin 1 and 2 on the CXCR3 WT and its mutants using a BRET based assay (Supplemental Information Table S7-S10, Fig. 7 and 8). Because only a few amino-acid substitutions resulted in differentiation between the β-arrestin 1 and 2 pathways, the general discussion of the observations applies for the both β-arrestin isomers. The exceptions will be pointed out explicitly. The rank order of functional affinities at wild type CXCR3 was similar as for the G protein activation.

The only deviation within the two investigated pathways at wild type CXCR3 was noted for the cooperativity between BD103 and CXCL10; the cooperativity was diminished 17-fold (β-arrestin 1) and 26-fold (β-arrestin 2), whereas the α value for G protein activation was approximately zero, and thus undistinguishable from competitive antagonism (Supplemental Information Tables S8 and S10). However, this was a probe-dependent effect because in both pathways BD103 showed a negative cooperativity towards CXCL11. Substitution of D186$^{4.60}$ for asparagine followed a similar trend as observed in the cAMP assay, by increasing the functional activities of cRAMX3 and BD064 to inhibit CXCL11-mediated β-arrestin 2 recruitment (6-fold and 9.5-fold, respectively, Supplemental Information

Table S7, S9 and Fig. 7I). However, the increase was not statistically significant in the β-arrestin 1 pathway. The D297$^{7.32}$N mutant showed no notable effect on cRAMX3 and BD064 to modulate CXCL11-induced recruitment of β-arrestins. However, for CXCL10- induced recruitment of β-arrestin 1 this mutation led to opposite effects on the functional activity of cRAMX3 between the G protein and β-arrestin 1 pathways with a 6-fold increase in the former and a 5-fold decrease in the latter (Fig. 8B). The resulting difference in functional activity (30-fold) indicates that this residue is an intersection point between G protein and β-arrestin 1 recruitment for cRAMX3. For BD103, the introduced asparagine improved the negative cooperativity exhibited towards CXCL10 in the β-arrestin pathway to such an extent that it reached an α value of approximately 0 undistinguishable from competitive antagonism (Fig. 8F), and the functional activity between BD103 and CXCL11 (8.5 and 5-fold, respectively β-arrestin 1 and β-arrestin 2). Further gains in the pK_b value of BD103 were detected at Y271$^{6.51}$F (6.5-fold) to modulate CXCL11 mediated β-arrestin 1 recruitment (Table 7 and 9). In contrast, W268$^{6.48}$F completely abolished the response of BD103 towards CXCL10 for β-arrestin 1 recruitment (Fig. 8H). Moreover, this effect was probe dependent as W286$^{6.48}$F had no effect on CXCL11-induced β-arrestin 1 recruitment. The other two modulators were mostly unaffected by theses residues except for the W268$^{6.48}$F mutant, which caused significantly reduced pK_b values for cRAMX3 and BD064 to inhibit CXCL10-mediated β-arrestin 1 recruitment (Fig. 8E). However, this effect could be also a reason for the dramatically reduced efficacy of CXCL10 and the resulting small measurement window. Variable reductions in functional affinity (5- to 19-fold) and cooperativity were noted for all three compounds to inhibit the CXCLL- and CXCL10-elicited response at the F131$^{3.32}$A mutant with BD103 having the most drastic effect (Fig. 7A-C). Interestingly, the influence of the mutation was less pronounced in the presence of CLXC10, especially in the β-arrestin 2 pathway, which indicates probe-dependence (Fig. 8A, D and G). The substitution of S304$^{7.39}$ for glutamic acid revealed a remarkably lower functional affinity of cRAMX3 to inhibit CXCL11 elicited recruitment of β-arrestin 1 (4-fold) and an even more drastic impact

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for the β-arrestin 2 pathway (10-fold). In contrast BD064 suffered the greatest losses in cooperativity leading to an almost complete abrogation of signaling (Fig. 7E). In addition, BD103 followed a similar trend with significant reductions in functional affinity and cooperativity, whereas this mutation had no influence on the modulator in the cAMP assay (Fig. 7F). The most profound results were seen at the Y308$^{7.43}$A mutant, where the inhibitory effect on CXCL11-mediated β-arrestin recruitment was totally abolished irrespective of the allosteric modulator tested. Again, the most drastic influence, switching the negative cooperativity to positive, was seen for BD103 (Fig. 7H). No α values between the modulators and CXCL10 could be derived from the results due to the low functional affinity and the precipitous dose-response curve (Fig. 8I). These findings are consistent with the results from the G protein activation experiments suggesting that these two residues likely contribute to a region in the network of interactions that govern the transmission of signaling for both pathways investigated. The pK_b values estimated obtained from the β-arrestin recruitment studies at the remaining mutants were not significantly different from the wild type CXCR3.

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Discussion

Allosteric ligands can robustly affect domain coupling and the conformational rearrangement upon chemokine activation of the chemokine receptors. Because the complex pharmacology of chemokines and their receptors might be a reason for the high attrition rate in the development of drugs that target chemokine receptors (Tschammer et al., 2014), it is essential to dissect molecular mechanisms that govern the interactions between allosteric modulators, chemokines and their receptors. With the aim to improve the understanding of the complex molecular mechanisms that govern the functions of CXCR3, we recently designed and characterized novel biased negative allosteric modulators of CXCR3, which elicit a bias in the inhibition of a given signaling pathway (G-protein-dependent signaling or recruitment of β-arrestin) and additionally tend to inhibit a specific pathway depending on the endogenous agonist used (Bernat et al., 2014; Bernat et al., 2015). In this work, we have rationalized molecular mechanisms that direct the effects of the biased negative allosteric modulators BD064 and BD103, and the unselective cRAMX3. Starting with a homology model (Milanos et al., 2016b) and docking of BD064 and BD103 we created a model that helped us to define the region suitable for the site-directed mutagenesis. By the combination of site-directed mutagenesis and detailed functional assays, we identified and categorized the contribution of various amino acids to the binding affinity and activity of investigated CXCR3 ligands. Obtained data were analyzed by the allosteric ternary complex model (ATCM), which is the simplest mechanism to describe allosteric interactions (Christopoulos and Kenakin, 2002). Although the general application of this model is to binding data, it can also be applied to functional data, but requires the additional assumption that the allosteric modulator does not perturb the signaling capacy of the receptor. When allosteric modulators change orthosteric ligand signaling efficacy in addition to, or instead of, any observed effects on binding affinity, the ATCM is insufficient (Christopoulos, 2002). For allosteric ligands that also act as agonists in their own right, an extended model is needed that consider the isomerization of a GPCR between active and inactive states (Hall, 2000); or for ligands that change

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kinetics of orthosteric ligand-receptor interactions (Avlani et al., 2004). Although the ATCM shows some limitations related to the correlation between parameters and the dependence of some of these parameteres on tissue effects such as receptor number, receptor coupling efficiency and amplification within functional assays, it can still be very useful in providing semi-quantitative estimates of allosteric effects (Keov et al., 2011). To our knowledge, this is a first comprehensive study of the interactions between the endogenous chemokines and biased negative allosteric inhibitors of CXCR3.

The mutation of aspartic acids D186$^{4.60}$ and D297$^{3.32}$ to asparagine abolished the probe-dependency previously displayed by BD064, which preferentially inhibits the activation of CXCR3 by CXCL10 (Bernat et al., 2015). This indicates that the aspartic acid contributes to a conformation of CXCR3 that allows BD064 to inhibit CXCL10 preferentially. The introduction of the asparagine abolished constraints responsible for the probe-dependency, resulting in an undistinguishable inhibition between CXCL11 and CXCL10. The mutations D186$^{4.60}$N and D297$^{3.32}$N also increased both the functional affinity of cRAMX3 to modulate CXCL10-mediated G protein activation and the cooperativity between BD103 and CXCL11. In contrast, only D186$^{4.60}$N had a similar enhancing effect on all three allosteric modulators to inhibit CXCL11-induced β-arrestin 2 recruitment. The mutation of residue D297$^{3.32}$ induced probe-dependence for cRAMX3 as the functional activity of cRAMX3 to inhibit CXCL10-mediated recruitment of β-arrestin 1 was dramatically decreased, whereas CXCL11-mediated recruitment of β-arrestin 1 was unaffected. In contrast, the inhibition of CXCL10-induced G protein activation by cRAMX3 was improved at this mutant. The resulting difference in functional affinity indicates that D297$^{3.32}$ functions as a molecular switch or gatekeeper, which constrains the recruitment of β-arrestin 1 and may serve as a key residue to trigger bias signaling between the two arrestin isoforms as well as G protein signaling. The asparagine introduced at this position yielded an enhanced cooperativity between BD103 and CXCL10 and higher functional affinity to activity to inhibit CXCL11-mediated β-arrestin recruitment without affecting the inhibition of G-protein activation.

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A highly conserved residue W268$^{6.48}$ is involved in the activation of the GPCRs (Schwartz et al., 2006). It was found that mutation of W248$^{6.48}$ into phenylalanine led to a G protein active, but β-arrestin inactive, and thus biased CCR5 conformation (Steen et al., 2013). In CXCR3, the mutation of W268$^{6.48}$ to phenylalanine caused variable probe-dependent effects on the modulators irrespective of the pathway investigated. Generally, the ability of the biased negative allosteric modulators BD064 and BD103 to inhibit CXCL10-mediated signaling was impaired; BD103 showed the most dramatic loss of function. Markedly, although the ability of cRAMX3 to inhibit CXCL11-mediated G protein activation was impaired, its ability to inhibit CXCL10-mediated G protein activation was not affected. According to our docking studies, the residue W268$^{6.48}$ does not interact directly with any of the ligands; as reported for other GPCRs (Schwartz et al., 2006), it steers between the active and inactive conformations of the GPCR. Our data show that in CXCR3, W268$^{6.48}$ is not a simple molecular on-off switch, but rather fine-tunes the responses, depending on the nature of a ligand and thus importantly contributes to the probe-dependency.

Another vital residue for the probe-dependency is F131$^{3.32}$. The mutation F131$^{3.32}$A impaired the ability of cRAMX3 and BD064 to inhibit CXCL11-induced G protein activation and β-arrestin recruitment. Noteworthy, in presence of CXCL10 the signaling of cRAMX3 and BD064 was mostly unchanged, which clearly indicates probe-dependent contribution of F131$^{3.32}$ to the overall signaling of CXCR3. Also, the F131$^{3.32}$A mutant did not impact BD103 to modulate G protein activation, however, it completely abolished the response of BD103 in the β-arrestin recruitment pathway irrespective of the chemokine used. This finding underscores the importance of F131$^{3.32}$ as the residue responsible for the biased inhibition of chemokine-induced signaling by BD103. The importance of this residue as the anchor point was also described for the structurally related ligand VUF10085, where the mutation to alanine significantly decreased the ability of VUF10085 to inhibit chemotactic response to CXCL11 (Nedjai et al., 2015). Similarly, the Y308$^{7.43}$A mutant rendered VUF10085 impotent, which agrees with

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our current findings. In the cAMP assay, the mutation Y308$^{7.43}$A diminished both the functional affinity and the cooperativity of cRAMX3 towards CXCL11, whereas only the functional affinity of cRAMX3 was reduced upon activation with CXCL10 indicating a specific implication of Y308$^{7.43}$ in the transmission of cooperativity between cRAMX3 and CXCL11. An even more striking effect was observed for BD064 where the Y308$^{7.43}$A mutant induced a complete loss of allosteric modulation of CXCL11-mediated G protein activation, although BD064 was still able to inhibit CXCL10-mediated response but with significant reductions in functional affinity and cooperativity. The importance of this residue was further confirmed by the finding that all three allosteric modulators elicited no discernible β-arrestin recruitment at the Y308$^{7.43}$A mutant. Likewise, substitution of S304$^{7.39}$ with glutamic acid substantially affected the ability of cRAMX3 and BD064 to modulate CXCL11- and CXCL10 induced G protein activation and β-arrestin recruitment. It is noteworthy that this residue plays a key role in the transmission of cooperativity between BD064 and either CXCL11 or CXCL10 as mutation to glutamic acid results in an almost complete abrogation of signaling. The inhibitory properties of BD103 were only influenced in the β-arrestin recruitment assay at S304$^{7.39}$E. Overall, all three mutations (F131$^{3.32}$A, Y308$^{7.43}$A and S304$^{7.39}$E) showed significant impact on the binding of BD064 and BD103 and affected the signaling of CXCL11 and CXCL10 significantly. Finally, they substantially influenced the functional properties of the negative allosteric modulators in all pathways investigated.

Besides the discovery of amino acids that contribute to the phenomenon of bias and probe-dependent signaling of the negative allosteric modulators, we further identified D186$^{4.60}$, W268$^{6.48}$ and S304$^{7.39}$ as key residues that are specifically involved in CXCL10-mediated β-arrestin recruitment but not G protein activation, as mutating them led to a G protein active but β-arrestin inactive conformation. Note that both CXCL11-mediated G protein activation and β-arrestin recruitment were mainly unaffected by the investigated mutations, which confirms different receptor conformations stabilized by each chemokine.

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In summary, we have identified key residues in the CXCR3 receptor that are involved in the binding, signaling and transmission of cooperativity between the three negative allosteric modulators and the endogenous chemokine ligands CXCL11 and CXCL10. We confirmed that BD064 and BD103 adopt some slightly different binding modes, which are in turn responsible for the probe-dependent inhibition of G protein–dependent or –independent signaling. The differential effects of mutations on either negative allosteric modulators or chemokines clearly indicate that CXCR3 adopts multiple active states stabilized by the bound ligand(s). The transmission of the functional response is triggered by diverse molecular switches responsible for the phenomenon of ligand-directed signaling. Our findings provide further understanding of the structural basis of allosteric modulation in the chemokine receptors with special emphasis on probe-dependence, and can help guide structure-based design and optimization of allosteric ligands with improved physiological properties.

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Author contributions

Participated in research design: Brox, Milanos, Saleh, Buschauer, Heinrich, Clark, Tschammer

Conducted experiments: Brox, Milanos, Saleh, Baumeister, Hofmann

Performed data analysis: Brox, Milanos, Saleh

Wrote or contributed to the writing of the manuscript: Brox, Milanos, Saleh, Clark, Tschammer

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Footnotes

This study was supported by the grant of German Research Foundation [GRK1910]. Prof. Dr. Armin Buschauer deceased in July 2017.

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Legends of Figures

Fig. 1. *Chemical structures of negative allosteric modulators investigated in the current study.*

Fig. 2. *Binding mode of BD064 in CXCR3.* (A) 2D representation of BD064-CXCR3 interactions. Suggested receptor interactions are shown as dashed lines. (B) 3D representation of binding interactions between BD064 and the CXCR3 receptor model. The ligand is shown in orange and side chains of proposed interacting residues are shown in turquoise.

Fig. 3. *Binding mode of BD103 in CXCR3.* (A) 2D representation of BD103-CXCR3 interactions. (B) 3D representation of binding interactions between BD103 and the CXCR3 receptor model. The ligand is shown in purple and side chains of proposed interacting residues are shown in turquoise. (C) Enlarged view of the proposed binding pocket of BD103 illustrating the "hot spot" formed by $Y205^{ECL}$, $D186^{4.60}$ and $R126^{5.39}$.

Fig. 4 *Effects of CXCR3 mutations on allosteric modulator affinity and cooperativity estimates.* Bars represent the differences in pK_b (left panel) or binding cooperativity value (right panel) of allosteric modulators relative to WT as derived from radioligand RAMX3 displacement experiments. Data represent the mean ± S.E.M. of three to four experiments performed in triplicate. ND, not determinable, *, significantly different from WT, $p < 0.05$, one-way ANOVA with Dunnett`s post- test.

Fig. 5. *Effects of CXCR3 mutations on CXCL11 and CXCL10 signaling.* The bars represent the difference in log(max/EC_{50}) estimates of CXCL11 (red) and CXCL10 (blue) relative to CXCR3 WT. Data represent the mean ± S.E.M. of at least three experiments performed in triplicates. * $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$ significantly different from WT, one-way ANOVA with Dunnett`s post-test.

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Fig. 6. *Change in CXCL10 activity vs. CXCL11 with mutations.* Each point in the radar chart represents the ΔΔLog(max/EC_{50}) value, which is the effect of each mutation on the selective agonist by setting the ΔΔLog(max/EC_{50}) = 0 for WT CXCR3.

Fig. 7. *Different effects of mutations on the ability of modulators to inhibit CXCL11- mediated G protein activation or β-arrestin 1 recruitment.* The ability of negative allosteric modulators to inhibit the CXCL11- mediated (EC_{80}) activation of CXCR3 WT and mutants was determined by cAMP BRET and β-Arrestin 1 BRET assays. Dose-response curves represent the mean ± S.E.M. of 2-4 experiments performed in triplicate.

Fig. 8. *Different effects of mutations on the ability of modulators to inhibit CXCL10- mediated G protein activation or β-arrestin 1 recruitment.* The ability of negative allosteric modulators to inhibit the CXCL10- mediated (EC_{80}) activation of CXCR3 WT and mutants was determined by cAMP BRET and β-Arrestin 1 BRET assays. Dose-response curves represent the mean ± S.E.M. of 2-4 experiments performed in triplicate.

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Figures

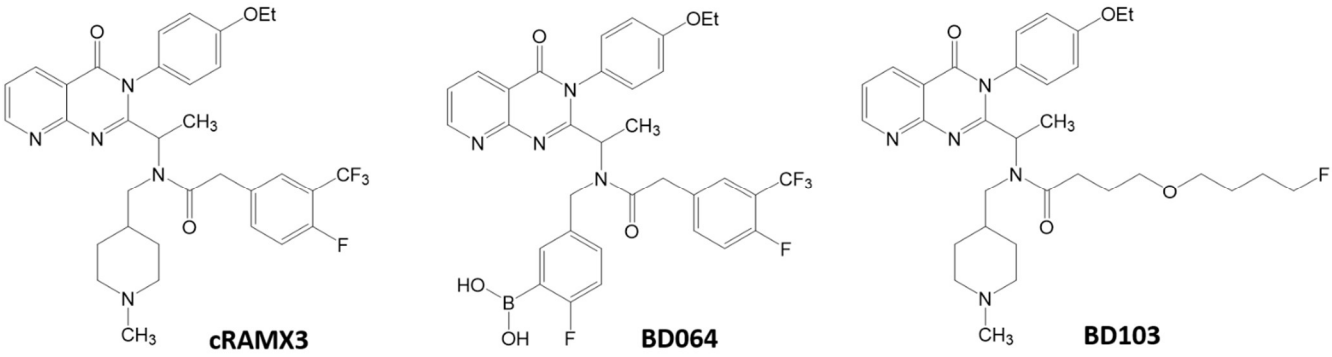

Figure 1

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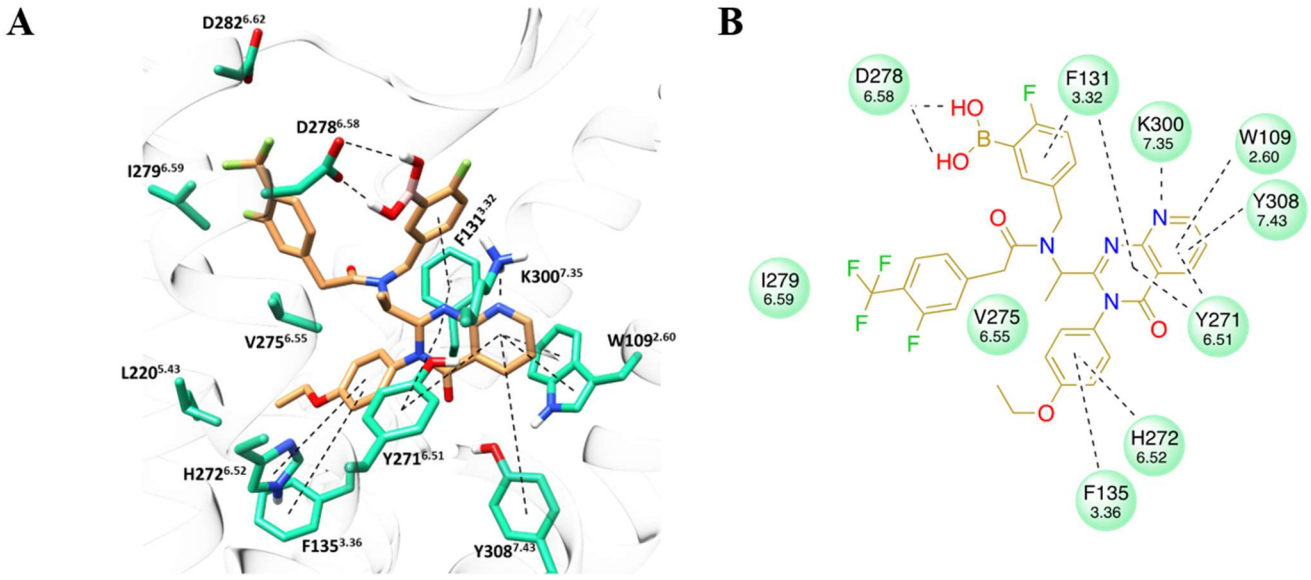

Figure 2

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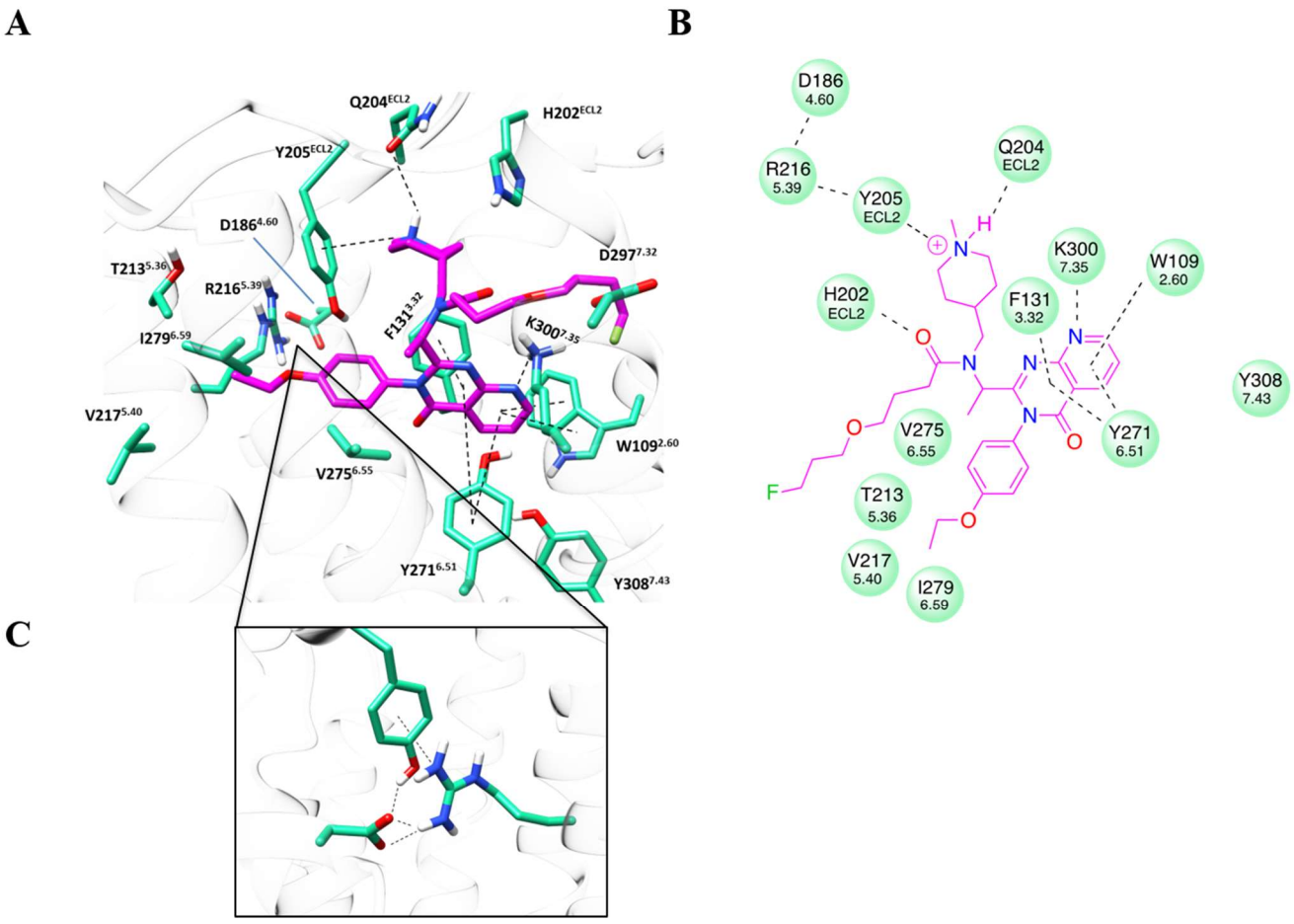

Figure 3

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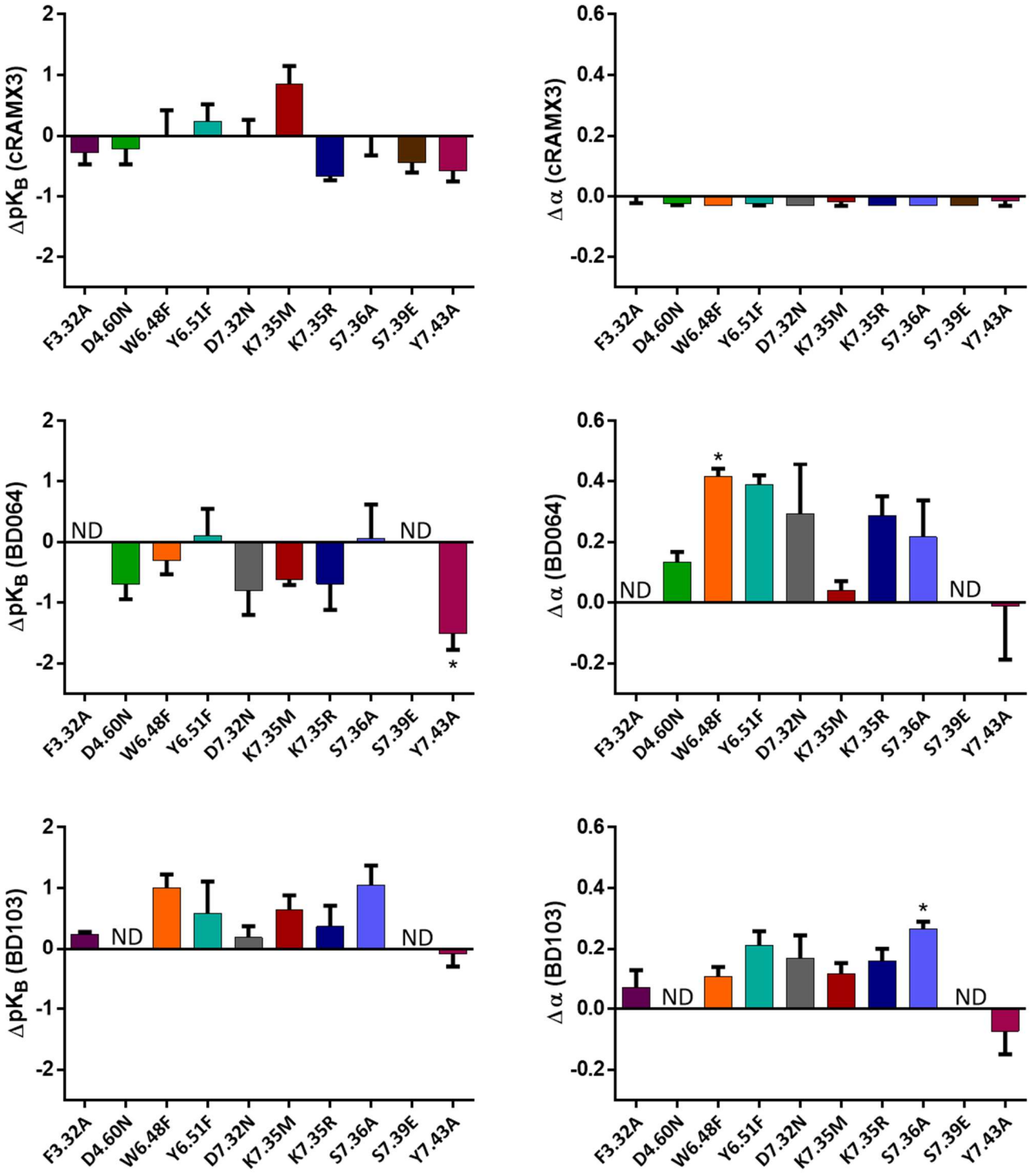

Figure 4

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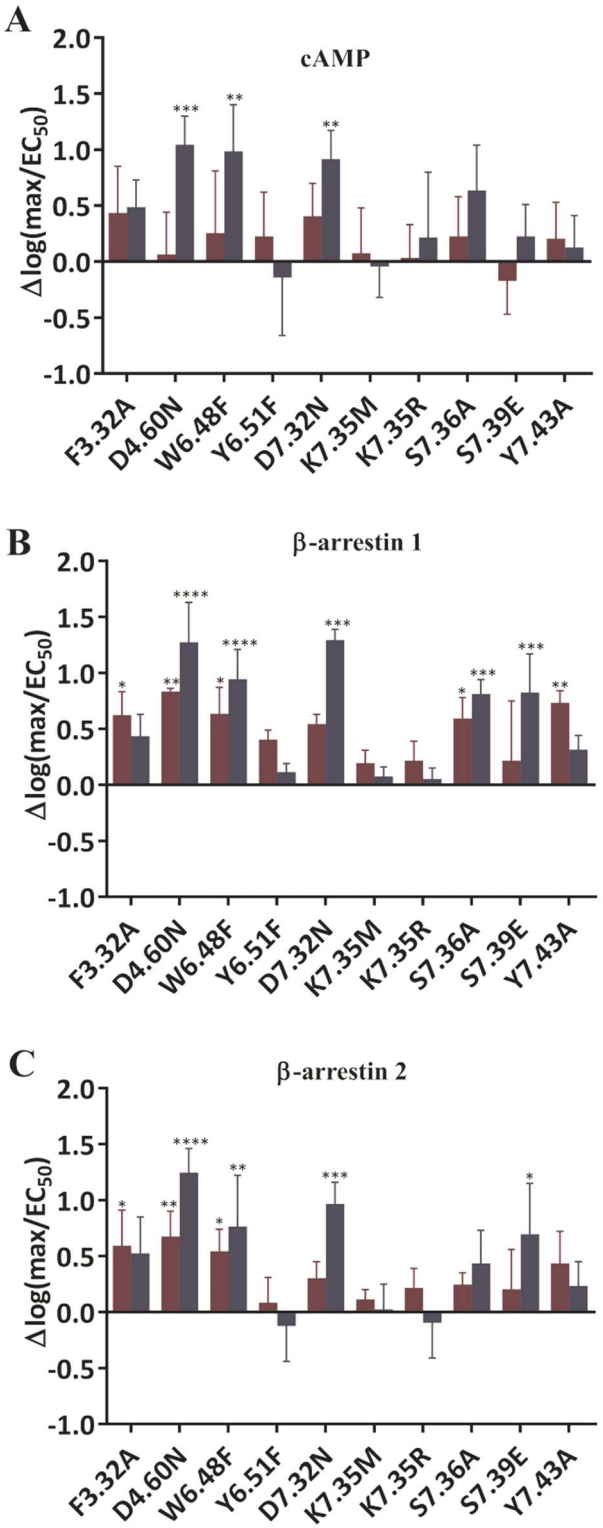

Figure 5

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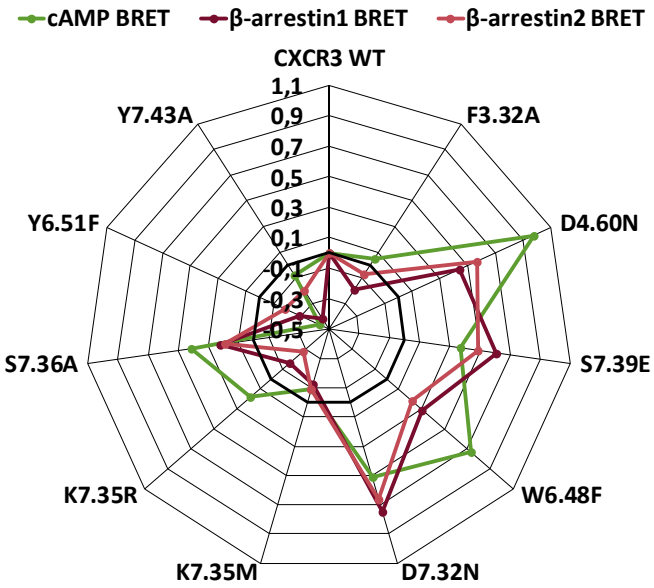

Figure 6

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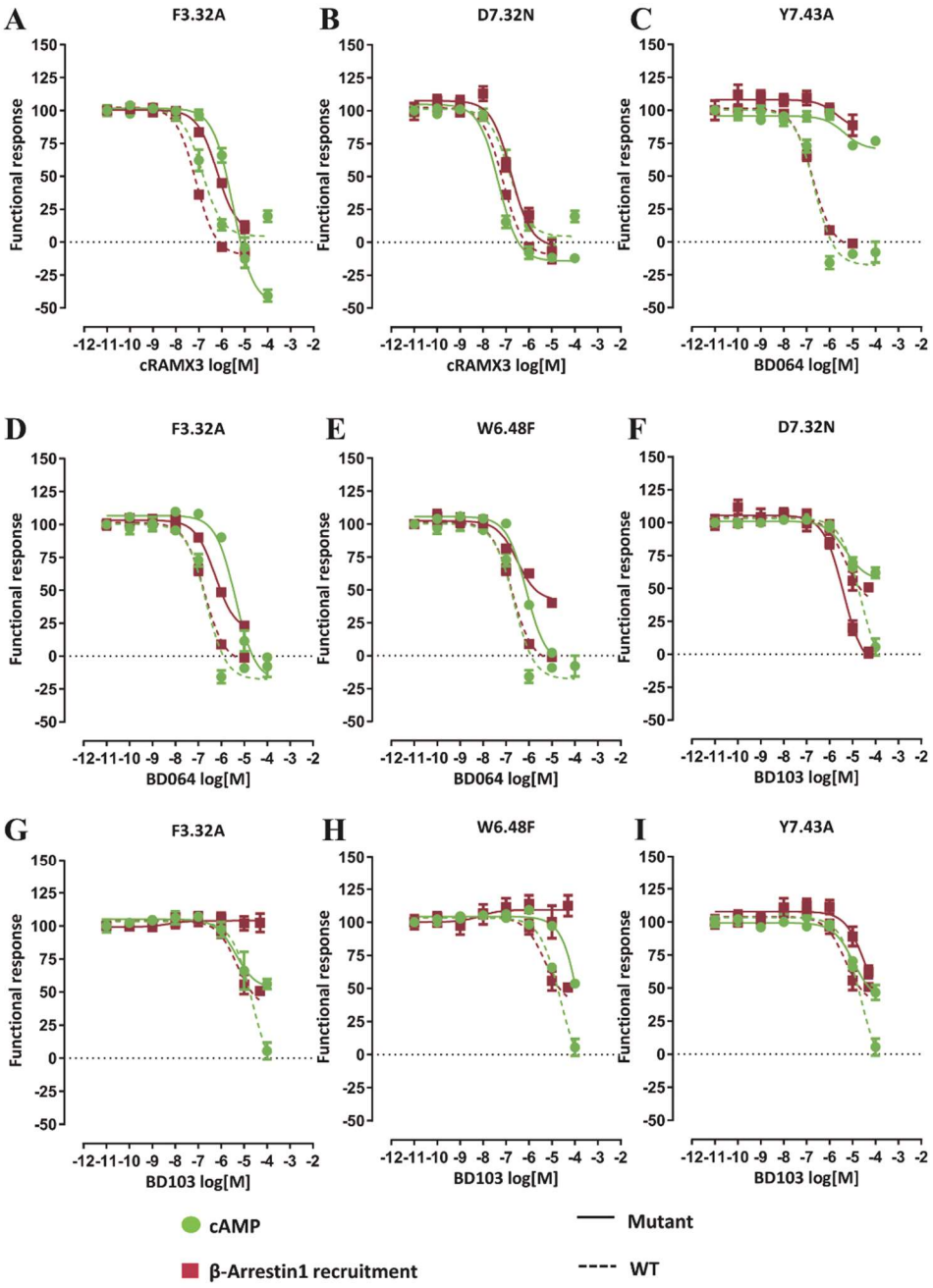

Figure 7

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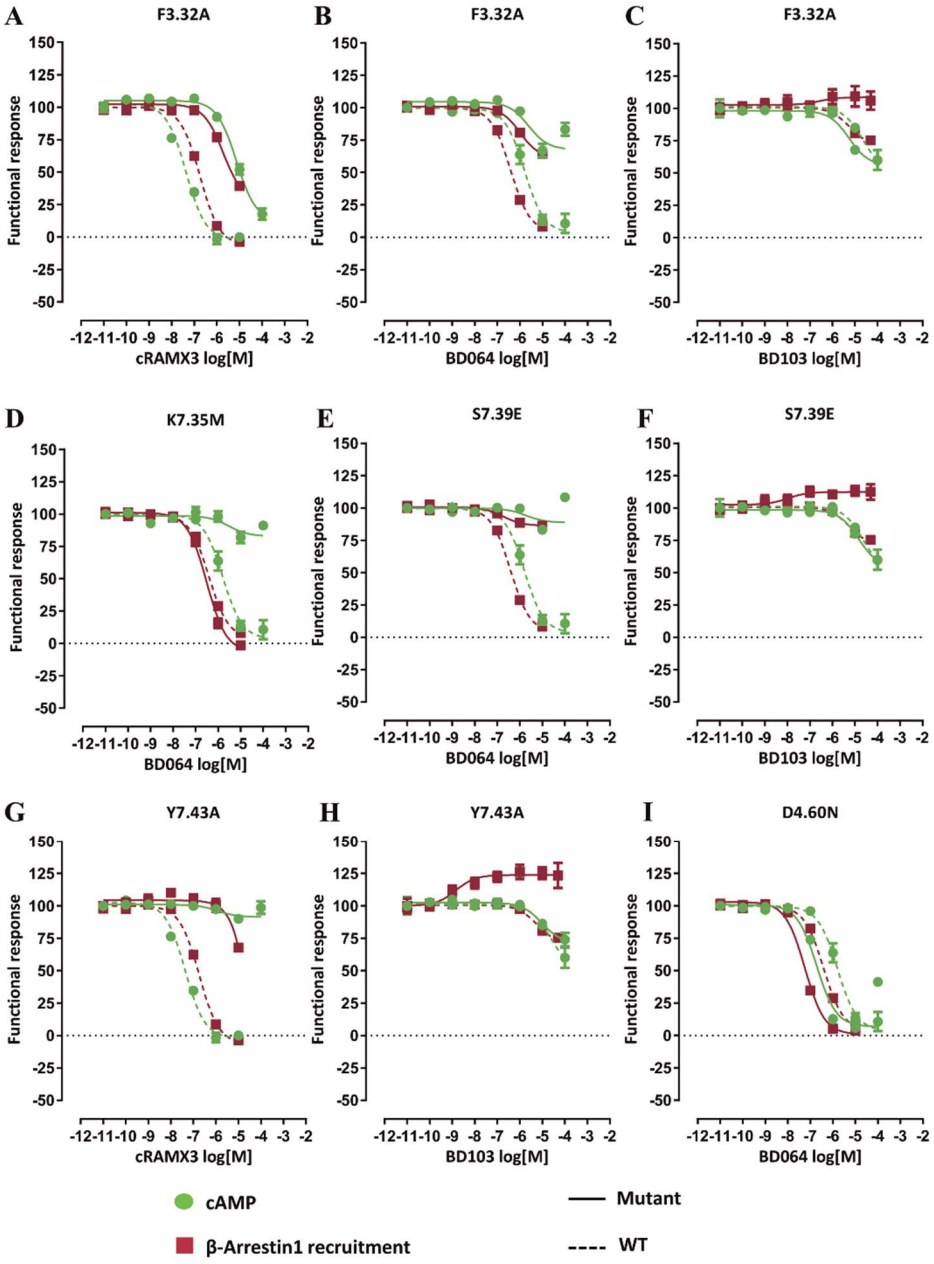

Figure 8