Fluorescent ligands targeting the intracellular allosteric binding site of the chemokine receptor CCR2

Lara Toy, Max E. Huber, Maximilian F. Schmidt, Dorothee Weikert, Matthias Schiedel*

Department of Chemistry and Pharmacy, Medicinal Chemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Nikolaus-Fiebiger-Straße 10, 91058 Erlangen, Germany
E-mail: matthias.schiedel@fau.de

ABSTRACT: Fluorescently labeled ligands are versatile molecular tools to study G protein-coupled receptors (GPCRs) and can be used for a range of different applications, including bioluminescence resonance energy transfer (BRET) assays. Here, we report the structure-based development of fluorescent ligands targeting the intracellular allosteric binding site (IABS) of the CC chemokine receptor 2 (CCR2), a class A GPCR that has been pursued as a drug target in oncology and inflammation. Starting from previously reported intracellular CCR2 antagonists, several tetramethylrhodamine (TAMRA) labeled CCR2 ligands were designed, synthesized and tested for their suitability as fluorescent reporters to probe binding to the IABS of CCR2. By means of these studies, we developed 14 as a fluorescent CCR2 ligand enabling cell-free as well as cellular NanoBRET-based binding studies in a non-isotopic and high-throughput manner. Further, we show that 14 can be used as a tool for fragment-based screening approaches as well. Thus, our small molecule-based fluorescent CCR2 ligand represents a promising tool for future studies of CCR2 pharmacology.

G protein-coupled receptors (GPCRs) are pharmaceutically highly relevant, as they are targeted by approximately one third of all available medications. The vast majority of the reported GPCR ligands binds to an orthosteric site located within the helical bundle, facing the extracellular side of the receptor. Apart from the orthosteric site, binding the endogenous ligand, an intracellular allosteric binding site (IABS) has been recently identified by X-ray co-crystallography for the chemokine receptors CCR2, CXCR2, CCR7, CCR9 as well as for the beta-2 adrenergic receptor (β2AR). Moreover, a druggable IABS has been suggested for several other GPCRs. Ligands targeting this allosteric binding site feature a new dual mechanism of specific GPCR modulation, which is characterized by a stabilization of the inactive receptor conformation and a steric blockage of intracellular transducer (G protein and/or β-arrestin) binding. Taking advantage of this new approach of GPCR antagonism is especially attractive for GPCR families, where the development of orthosteric antagonists only showed very limited therapeutic success, such as the chemokine receptors.

CCR2-mediated signaling has been associated with the pathogenesis of various diseases, including atherosclerosis, multiple sclerosis, amyotrophic lateral sclerosis, severe acute respiratory syndrome (SARS), coronavirus disease 2019 (COVID-19), and cancer. A special interest in CCR2 antagonists was triggered by highly promising preclinical results, suggesting their application for the treatment of pancreatic ductal adenocarcinomas (PDAC). However, several clinical trials investigating the therapeutic potential of orthosteric CCR2 antagonists for the treatment of PDAC and other CCR2-related diseases ultimately failed to provide an approved drug. By providing an alternative approach for CCR2 inhibition, intracellular allosteric CCR2 antagonists are therefore highly interesting compounds for future clinical investigation.

Several intracellular allosteric CCR2 antagonists, including biaryl sulfonamides (e.g. SD-24 (1) and 2) and pyrrolones (e.g. CCR2-RA (4)) have already been identified and chemically optimized in the absence of any detailed structural information on intracellular CCR2 antagonism (Chart 1). With the disclosure of the crystal structure of CCR2 in complex with the CCR2-RA eutomer 4-[R], Zheng et al. laid the foundation for the structure-based development of further intracellular CCR2 antagonists, such as the covalent biaryl sulfonamide analogue 3. Besides structural insights on receptor-ligand interactions, molecular tools that enable an unambiguous identification and characterization of intracellular CCR2 ligands are of utmost importance for drug discovery campaigns in this field. With the radioligand [3H]CCR2-RA, Zweemer et al. have therefore reported a highly valuable molecular tool that can be utilized to detect binding to the IABS of CCR2.

<table>
<thead>
<tr>
<th>Biaryl sulfonamides:</th>
<th>R1</th>
<th>R2</th>
<th>Kd [nM]</th>
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<tbody>
<tr>
<td>1 (SD-24)</td>
<td>CH</td>
<td>-Cl</td>
<td>-COOH (ortho)</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>-CF₃</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CH</td>
<td>-CF₃</td>
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<tr>
<th>Pyrrolones:</th>
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<th>Kd [nM]</th>
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<tr>
<td>4 (CCR2-RA)</td>
<td>-Cl</td>
<td>11 nM²</td>
</tr>
<tr>
<td>5</td>
<td>-Br</td>
<td>6 nM²</td>
</tr>
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Chart 1. Chemical structures of selected intracellular allosteric antagonist (1-5). Biological activity (Kd values) as reported by Peace et al., Wang et al., Ortiz Zacharias et al. (2021), and Ortiz Zacharias et al. (2018).
In more recent studies, the eutomer \([{}^3\text{H}]\text{CCR2-RA-}[R]\) was used instead of the racemic mixture. This molecular tool has frequently been applied and has successfully guided the structure-based development of CCR2 antagonists such as 3 and 5.\(^{20,22}\) However, radioligand binding assays are accompanied by several drawbacks, such as high infrastructure requirements according to radiation protection measures, the production of radioactive waste, and a laborious (heterogeneous) assay protocol including washing steps, to remove the unbound radioligand prior to assay readout. The latter is also the reason why radioligand binding assays are often not well-suited for a continuous readout or the detection of low affinity binders, such as fragments. Recently, we reported the development of a fluorescent tracer targeting the IABS of CCR9, which was successfully applied for binding studies using the non-isotopic NanoBRET technology.\(^{23}\) Herein, we aimed at developing a fluorescently labeled intracellular CCR2 ligand, in order to provide a molecular tool that allows to study direct binding to the IABS of CCR2 in a non-isotopic manner.

RESULTS

For the design of fluorescently labeled intracellular CCR2 ligands, we first identified suitable positions at the biaryl sulfonamide- and pyrrolone-based pharmacophores, respectively, that allow an installation of a linker unit. Based on the reported cocrystal structure of 4-[R] in complex with CCR2,\(^1\) we performed molecular docking with several CCR2 ligand-linker-conjugates (Figure S1). In the case of the pyrrolone-based scaffold, these studies indicated that the Cα-position of the acetyl group of 4 (Chart 1) is suitable for the installation of a linker (Figure 1).

As the bromo analogue (5) was reported to have an increased CCR2 affinity compared to 4 (see Chart 1), fluorescently labeled derivatives of 5 were included in our study. For the biaryl sulfonamide-based CCR2 antagonists, the development of 2 and 3 already provided evidence that the attachment of a functional label via amide bond formation in the para position is well tolerated (Chart 1). This was also supported by our docking results for sulfonamide-based CCR2 ligand-linker-conjugates (Figure 1 and S1). Thus, we mainly focused on para-substituted analogues for the development of our biaryl sulfonamide-based fluorescent CCR2 ligands. As high docking scores were also obtained for ortho-substituted analogues (Figure S1), we considered compounds with this substitution pattern as well. Moreover, our docking experiments revealed promising properties for triazole containing linkers, which enable a straightforward conjugation with a fluorophore by means of Cu(I)-catalyzed Huisgen cycloaddition.\(^{24}\)

Following these design suggestions, we established synthetic approaches towards the pyrrolone-based fluorescent CCR2 ligands 8-9 as well as the biaryl sulfonamide-based fluorescent CCR2 ligands 10-16 (Scheme 1). Our synthetic approaches were directed towards a late stage conjugation of the respective CCR2 ligand with a cell-permeable TAMRA-based fluorophore.\(^{24}\) The synthesis of the pyrrolone-based fluorescent CCR2 ligands (8-9, Scheme 1A) was initiated by an Sn2 reaction of 5-chloropentan-2-one with sodium azide to form 5-azidopentan-2-one (17), which was used as a starting material in a Claisen condensation to yield the β-keto ester (18). For the synthesis of the pyrrolone-based pharmacophore, we used 18, cyclohexanecarbaldehyde, and an aniline analogue as substrates for a previously reported one-pot three-component reaction.\(^{22}\) Finally, the azido-functionalized pyrrolones (19-20) were conjugated with 6-TAMRA alkyne by means of click chemistry to yield the pyrrolone-based fluorescent CCR2 ligands 8 and 9. For the synthesis of the biaryl sulfonamide-based fluorescent CCR2 ligands (10-16, Scheme 1B/C), we adapted previously published procedures.\(^{17,20}\) In a first step, a fluorinated benzene or pyridine derivative was reacted with ortho- or para-hydroxy benzoic acid methyl ester to generate the biaryl ethers (21-22, 33) by means of a nucleophilic aromatic substitution. Reduction of the aromatic nitro group using tin(II) chloride gave the primary aromatic amines (23-24, 34). A sulfonylation of the primary aromatic amines resulted in the sulfonamides (37-40, Supporting Information), which were subjected to saponification with lithium hydroxide to yield the benzoic acid analogues (1, 25-27). Subsequently, the released carboxylic acid was used for the installation of a linker with a clickable moiety via amide coupling. The obtained amides (28-32, 35-36) were then finally conjugated with the respective functionalized TAMRA fluorophores\(^{25}\) via Cu-catalyzed azide-alkyne cycloaddition to yield the biaryl sulfonamide-based fluorescent CCR2 ligands (10-16).

Figure 1. Design of fluorescent ligands targeting the intracellular allosteric binding site of CCR2. A) Chemical structure of CCR2 ligand-linker-conjugates (6-7) that were identified by molecular docking as suitable templates for the design of fluorescent ligands. B) Overlay of the reported binding mode of 4-[R] (PDB ID: 5T1A)\(^1\) with the predicted binding modes for 6 and 7.
Scheme 1. Synthesis of fluorescent ligands targeting the intracellular allosteric binding site of CCR2. A) Synthesis of pyrroline-based fluorescent CCR2 ligands 8-9. Reagents and Conditions: a) NaN₃, DMSO, 70°C, 24 h, 31% yield; b) diethyl oxalate, NaOEt, EtOH, 30% yield; c) cyclohexanecarbaldehyde, 2-fluoroaniline analogue, AcOH, 95°C, 4 h, 32–36% yield; d) 6-TAMRA-alkyne (42, see Supporting Information), 0.1 M CuSO₄ solution, 0.1 M sodium ascorbate solution, TBDTA, water/tBuOH/DMF mixture (1:1:1 (v/v)), rt., 4–16 h, 22–38% yield. B/C) Synthesis of biaryl sulfonamide-based fluorescent CCR2 ligands with a para- (see B) or ortho-substitution pattern (see C). CCR2 ligands were either conjugated with a 6-TAMRA- (10-12, 14-15) or 5-TAMRA-based fluorophore (13, 16). Reagents and Conditions: e) K₂CO₃ or Cs₂CO₃, DMF, 60°C, 2–4 h, 84–94% yield; f) SnCl₂, EtOH, 0°C to 50°C, 3–20 h, 84–95% yield; g) i: benzenesulfonyl chloride analogue, pyridine/CH₂Cl₂, reflux, 4-5 h, 58–95% yield; ii: LiOH, THF/H₂O (1:1 (v/v)), 21–40°C, 4-18 h, 92–97%; h) for 28, 29, 35: EDC, HOBT, DIPEA, azido-PEG₂-amine (41, see Supporting Information, Scheme S1), DMF, rt., 2–24 h, 70-82% yield; for 30: TBTU, DIPEA, azido-PEG₂-amine (41, see Supporting Information), DMF, rt. 2 h, 42% yield; i) 6-TAMRA-alkyne (42), 0.1 M CuSO₄ solution, 0.1 M sodium ascorbate solution, TBDTA, water/tBuOH/DMF mixture (1:1:1 (v/v)), rt., 2–16 h, yield 23-78%; j) for 31 and 36: propargyl amine, EDC, HOBT, DIPEA, DMF, rt., 2 h, 68–92% yield; for 32: propargyl amine, TBTU, DIPEA, DMF, 1 h, 0°C to rt., 98% yield; k) 5-TAMRA-azide (for 13, 16) or 6-TAMRA-PEG₂-azide (43) (see Supporting Information) for 14, 0.1 M CuSO₄ solution, 0.1 M sodium ascorbate solution, TBDTA, water/tBuOH/DMF mixture (1:1:1 (v/v)), rt., 4–16 h, 32–61% yield.
In order to evaluate the binding affinities of the synthesized fluorescent CCR2 ligands, we developed a NanoBRET-based binding assay (Figure 2 and S2A-G). Therefore, we fused CCR2 at its intracellular C-terminus to a small and bright luciferase variant (nanoluciferase, Nluc). Successful surface expression of CCR2-Nluc fusion proteins was confirmed by ELISA directed against an N-terminal HA-tag (Figure S2D). In saturation binding experiments using membranes from HEK293T cells transiently expressing Nluc-tagged CCR2 with a GSSG linker between the C-terminus of CCR2 and the Nluc-tag (hereafter referred to as CCR2_GSSG_Nluc), 14 showed the highest binding affinity among the tested ligands, with a $K_d$ value of 266 ± 28 nM (Table 1, Figure 2B and S2F-G). The obtained affinity data indicates that our approach for the design of fluorescently labeled intracellular CCR2 ligands was most successful, when the biaryl sulfonamide-based pharmacophore of 2 was used as a CCR2 binding unit. The difference in affinity between 12 and 14 might be rationalized by additional polar interactions of the triazole moiety with polar amino acids at the entrance of the intracellular allosteric binding pocket of CCR2 (see Figure 1B). Fluorescent ligands based on the biaryl sulfonamide-based pharmacophore of 1 (10, 13, 15, 16) showed reduced affinities, especially when the ether and benzamide substituents were placed in ortho position to each other (15-16). Low affinities were also detected for our pyrrolone-based fluorescent ligands (8-9).

Table 1. $K_d$ values of fluorescent CCR2 ligands determined in NanoBRET-based saturation experiments using CCR2_GSSG_Nluc membranes. For saturation curves see Figures 2B and S2F.

<table>
<thead>
<tr>
<th>compound</th>
<th>$K_d$ [nM] (N number)</th>
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<tbody>
<tr>
<td>8</td>
<td>1720 ± 360 (2)</td>
</tr>
<tr>
<td>9</td>
<td>2420 ± 140 (2)</td>
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<tr>
<td>10</td>
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<tr>
<td>11</td>
<td>679 ± 83 (2)</td>
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<td>13</td>
<td>940 ± 12 (2)</td>
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<tr>
<td>14</td>
<td>266 ± 28 (5)</td>
</tr>
<tr>
<td>15</td>
<td>5450 ± 160 (2)</td>
</tr>
<tr>
<td>16</td>
<td>2610 ± 130 (2)</td>
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</table>

Figure 2. NanoBRET binding assay for the IABS of CCR2. (A) Cartoon representation of the NanoBRET strategy to detect binding to the IABS of CCR2. (B) Saturation binding curve of 14 in a cell-free NanoBRET-based experiment using CCR2_GSSG_Nluc membranes (mean ± SD, triplicate measurement, n = 5). (C) Representative association (left) and dissociation (right) curves with 14 (650 nM) using CCR2_GSSG_Nluc membranes. (D) Competition binding curves for SD-24 (1; green) and CCR2-RA (4; blue) obtained with 14 (650 nM) and CCR2_GSSG_Nluc membranes (mean ± SEM, triplicate measurement, n = 3). (E) Chemical structures and competition binding curves for 14-related ligands 27 (purple) and 44 (red) obtained with 14 (650 nM) and CCR2_GSSG_Nluc membranes (mean ± SEM, triplicate measurement, n ≥ 3). The synthesis of 44 is outlined in the Supporting Information.
Kinetic binding studies with 14 revealed a very fast association of the ligand-receptor-complex by exhibiting a rate constant of $k_{\text{on}} = 6.00 \pm 0.39 \cdot 10^8 \text{M}^{-1} \cdot \text{min}^{-1}$ (Figure 2C). With a rate constant of $k_{\text{off}} = 0.101 \pm 0.005 \text{min}^{-1}$ and a residence time of $t_\text{r} = 9.93 \pm 0.43 \text{min}$, the dissociation of the ligand-receptor-complex was detected to be fast as well. This is in contrast to a recently reported fluorescent ligand targeting the IABS of the related chemokine receptor CCR9, which showed a much longer residence time ($t_\text{r} = 90 \text{min}$). The difference between the resulting kinetic $K_i$ value of 16.8 ± 0.1 nM and the equilibrium $K_i$ value of 266 ± 28 nM can be rationalized by the very fast association of ligand-receptor-complex, which mainly takes place in the first seconds of co-incubation and can therefore only be roughly estimated by the applied assay setup.

Next, we aimed to investigate the suitability of 14 as a molecular tool to study the binding of non-fluorescent ligands to the IABS of CCR2. Therefore, we set up displacement experiments with 14 and the known intracellular CCR2 antagonists SD-24 (1) and CCR2-RA (4). In agreement with previously reported nanomolar affinities, we obtained $K_i$ values of 28.4 ± 3.8 nM for 1 and 17.2 ± 2.6 nM for 4 (Figure 2D). To rationalize the design of our fluorescent CCR2 ligand 14 in a retrospective manner, we synthesized and tested 27 as the CCR2 binding unit of 14 as well as the 14-related ligand-linker-conjugate 44 (Figure 2E). Using our displacement assay, we detected nanomolar CCR2 affinities for 27 ($K_i = 6.22 \pm 1.33 \text{nM}$) and 44 ($K_i = 98.2 \pm 6.2 \text{nM}$), thereby corroborating the structure-based design of 14.

Furthermore, we tested the suitability of 14 as a molecular tool for future fragment-based drug discovery/screening approaches.

As fragment screening is usually associated with high compound concentrations and thus high amounts of DMSO as a co-solvent, we first investigated the DMSO tolerance of our NanoBRET-based CCR2 binding assay. Therefore, $K_i$ values for 4 were determined at different concentrations of DMSO. The obtained results, illustrated in Figure S3, show that the assay setup tolerates even high DMSO concentrations up to 10% (v/v). Subsequently, we tested several fragments of intracellular CCR2 ligands (e.g. SD-24 (1) and 27) for their ability to compete with 14 for intracellular CCR2 binding (Figure 3). As the four fragments that showed the highest CCR2 binding affinity harbour a biaryl sulfonamide substructure, our results highlight the importance of this substructure for intracellular CCR2 binding from a fragment-based perspective.

After having shown that our fluorescent CCR2 ligand 14 is a suitable tool to study binding of high to low affinity binders to the IABS of CCR2 in a cell-free assay setup, we were curious to see whether we could also use this approach in a live cell environment. In general, the assessment of the interactions between a drug and its protein target in a physiologically relevant cellular environment is a critical step in preclinical drug discovery. This step, also referred to as cellular target engagement, is highly important for successfully delivering compounds with the desired biological and ultimately clinical effects, as the on-target activity of small molecules can be affected significantly when transitioning from a cell-free to a cellular environment. A loss of activity in a cellular environment can be attributed to various factors, for example low cell permeability, compound efflux, or off-target protein binding. For our cell-based assay setup, we used live HEK293T cells transiently expressing a Nluc-tagged CCR2 with no linker between the C-terminus of CCR2 and the Nluc-tag (hereafter referred to as CCR2_Nluc), as this construct resulted in a larger and more robust assay window compared to CCR2_GSSG_Nluc under cellular conditions (Figure S4). With this cell-based assay setup, a $K_i$ value of 114.3 ± 22.0 nM for 14 was determined (Figure 4A), demonstrating that 14 is able to pass the cell membrane and bind to CCR2 on the intracellular side of the receptor. In a cell-based displacement assay, we detected low nanomolar $K_i$ values for literature-known intracellular CCR2 antagonists SD-24 (1) ($K_i = 8.50 \pm 2.49 \text{nM}$) and CCR2-RA (4) ($K_i = 4.20 \pm 1.39 \text{nM}$), see Figure 4B. These results are consistent with the affinities detected in the cell-free setup (Fig. 2D) as well as with the rank order determined in a cellular assay system ($EC_{50} = 236 \text{nM}$ for SD-24 (1) and $EC_{90} = 93 \text{nM}$ for CCR2-RA-[R] $EC_{50}$ for CCR2-RA-[R] (4-[R]) in a cellular IP turnover activation assay). For 27 and 44, we detected a cellular CCR2 $K_i$ value of 4.40 ± 0.96 nM and 55.5 ± 7.6 nM, respectively. Overall, results from live-cell NanoBRET and membrane-based experiments were in good agreement with each other. Thus, our fluorescent CCR2 ligand 14 is a valuable tool to study binding to the IABS of CCR2 in a cellular environment. Besides providing mere binding affinity, our cell-based assay allows initial insights into pharmacokinetic properties, especially membrane permeability. This is of utmost importance as the IABS represents an intracellular target site and ligands targeting this site should feature appropriate pharmacokinetics, especially membrane permeability, to be considered as suitable candidates for further development.
studies targeting the IABS of CCR2. Thus, a molecular tool to study cellular target engagement for the IABS can be used as a molecular tool to study cellular target engagement for the IABS of CCR2. This tool enabled equilibrium as well as kinetic binding studies via the NanoBRET technique. In a displacement setup, 14 was used for the detection of high affinity intracellular CCR2 antagonists as well as for the identification of low molecular weight ligands (fragments). Due to its straightforward homogeneous assay protocol, our membrane-based NanoBRET displacement assay is suitable for high-throughput screening approaches. In addition, we show that 14 can be used as a molecular tool to study cellular target engagement for the IABS of CCR2. Thus, 14 represents a promising tool for future studies targeting the IABS of CCR2.

ASSOCIATED CONTENT
Supporting Information. The Supporting Information including experimental procedures for compound synthesis, biological tests, computational methods, as well as Supplementary Figures, Supplementary Schemes, NMR spectra, and HPLC chromatograms is available free of charge via ChemRxiv.

REFERENCES


Table of Contents artwork

CCR2

Nluc

extracellular

intracellular

Fluorescence

BRET

TAMRA fluorophore

K_d(CCR2) = 205 nM (membranes)
K_d(Nluc) = 114 nM (in cells)