Structural analysis of CXCR4 – Antagonist interactions using saturation-transfer double-difference NMR

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CXCR4 is a GPCR involved in leukocyte trafficking. Small molecule antagonists of the receptor may treat inflammatory disease, cancer and HIV. Here we probe the binding of a tetrahydroisoquinoline-based antagonist (TIQ-10) to CXCR4 using saturation transfer double-difference (STDD) NMR. STDD spectra were acquired using extracts from Chinese Hamster Ovary cells expressing membrane-embedded CXCR4. The experiments demonstrate competitive binding between TIQ-10 and established antagonists and provide the TIQ-10 – CXCR4 binding epitope. Molecular modeling of TIQ-10 into the binding pocket provides a pose consistent with STDD-derived interactions. This study paves the way for future investigations of GPCR-ligand interactions in a biological milieu for use in chemical biology, biochemistry, structural biology, and rational drug design.

1. Introduction

G protein-coupled receptors (GPCRs) are dimeric proteins localized on the surface of cells that bind extracellular ligands to initiate intracellular signaling [1,2]. GPCR-targeting small molecules modulate cellular pharmacology and make up 27% of approved drugs [3]. Chemokine receptors are class A GPCRs expressed on leukocytes that regulate chemotaxis via chemokine ligand binding [4]. This role implicates chemokine receptors in inflammatory diseases and cancer metastasis. Over 20 different chemokine receptors interact with almost 50 different chemokine ligands [5], but despite two decades of effort, there are only two FDA-approved chemokine receptor drugs [6]. It follows that improved understanding of small molecule binding to chemokine receptors would facilitate rational design of next-generation drugs. However, obtaining GPCR-ligand structural information remains a challenging endeavor as not all complexes are amenable to crystallization [7]. Recent advances in protein engineering have created GPCRs with unnatural mutations and protein fusions amenable to crystallographic analysis [8], but these mutant constructs are incapable of native signaling [9]. Even with available crystal structures, chemokine receptors remain difficult to model due to diverse small molecule ligands and expansive binding sites [10].

Saturation transfer difference (STD) NMR has emerged as a tool to probe GPCR-ligand interactions and complement crystallography and computational modeling [11]. The technique requires a small amount of protein and a high concentration of weak binding ligand (K_d > 100 nM, assuming diffusion-limited on-rates). Selective irradiation of the macromolecule permits magnetization transfer onto the ligand that rapidly dissociates maintaining the non-equilibrium nuclear spin populations. The resulting STD-NMR spectrum reveals proton signals with relative intensities reflecting points of close contact with the protein (the binding epitope) [12,13]. Generally, more intense signals arise from ligand protons in close contact with the protein. STD-NMR can be used with complex protein sample mixtures (like membrane extracts [14,15] or whole cells [16]) by subtracting a reference spectrum from the original to deliver the saturation-transfer double-difference (STDD) NMR spectrum [17,18]. Integration of STDD spectra allows mapping of membrane protein - ligand binding recognition elements within living systems.

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CXR4 is a membrane-bound chemokine receptor that binds the ligand CXCL12/SDF-1α to regulate migration and adhesion of leukocytes [19]. This receptor is broadly expressed on stem cells and is up-regulated in over 20 different types of cancer making CXCR4 a drug target for oncology and inflammation [20]. CXCR4 is also recruited by T-tropic HIV for viral entry, and small molecules that bind to CXCR4 exhibit antiviral activity [21]. AMD3100 (Plerixafor, Fig. 1) is a CXCR4 antagonist that is approved for stem cell mobilization, but due to toxicity, the drug is not a viable long-term solution for chemokine-related disease intervention [22].

We recently reported a tetrahydroisoquinoline (TIQ)-based series of CXCR4 antagonists with TIQ-15 being the lead compound (Fig. 1) [23]. Atomistic insight into the TIQ – CXCR4 complex would aid in optimization of this series, but the lead compound is somewhat too potent for STDD NMR studies. Thus, a 1000-fold weaker antagonist composed of an aniline bottom TIQ ring (TIQ-10, Fig. 1) was selected as a probe. Here we present an STDD-guided structural analysis of TIQ-10 binding to CXCR4 in which we arrive at a peptide-bound CXCR4-CVX15 crystal structure (pdb 3OE0) [26] was prepared using the Protein Preparation Wizard with hydrogen bonds optimized. A docking grid was generated using Glide [27] centered on the Arg2 – Nal3 residues on the CVX15 peptide. Low-energy conformers of TIQ-10 were generated using MacroModel [28], and these conformers were docked flexibly into the grid. The most favorable pose is presented and discussed.

2. Materials and methods

TIQ-10 was synthesized as previously described [23].

2.1. NMR sample preparation

CHO-Glo cells were stably transfected with human CXCR4 cDNA encoded in pIRE5-AcGFP1. CXCR4-expressing CHO cells were grown to 80% confluency on proline-supplemented Dulbecco’s Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Steptavidin. Cells were removed from monolayer using 0.05% EDTA then harvested by gentle centrifugation (700 rpm, 7 min). The cells were resuspended in 20 mL PBS then homogenized, and membranes were precipitated by ultracentrifuge (20 k rpm, 20 min, 4°C). The membranes were washed with cold PBS then resuspended in 1 mL 100% deuterated PBS. A stock solution of TIQ-10 in DMSO-d6 (30 mM) was added to deuterated PBS to a concentration of 3 mM then filtered. The NMR samples consisted of 100 μL of resuspended membrane extracts, 100 μL of TIQ-10 stock, and 100 μL of stock buffer/3 mM AMD3100/3 mM TIQ-15 all in 100% deuterated PBS (pH = 7.4) to a final volume of 300 μL.

2.2. NMR data collection

Fig. 1. Chemical structures of AMD3100, TIQ-15 and TIQ-10. The aromatic proton numbering scheme for TIQ-10 sites H1–H3 on the top THQ ring and H4–H7 on the bottom benzyl ring.

Spectra were collected using a Varian 600 MHz NMR equipped with an HCN triple-detection cryoprobe at 25°C (UGA CCCR). VNMRJ v. 4.0 was used to collect the data, and the data were processed using ACD Labs software (v. 12.01 Academic Version). STD spectra were collected using exactly the same parameters with the Saturation Transfer 1D-2 pulse sequence available in the Varian Biopack expansion with in-cycle subtraction. A number of 50 ms Gaussian pulses were applied in sequence to a saturation time presented in the text, and these pulses were applied at the on-resonance frequency of −0.5 ppm and off-resonance frequency of −20 ppm. Broad signals arising from the membranes were removed using a 10 msec trim pulse with a power of 46 dB. 128 scans were averaged with a 1 s acquisition time at 12 k data points (with 4 steady-state pre-scans) and a delay of 3 s to ensure complete relaxation of protons between scans. A 2 Hz exponential line-broadening function was applied to the free induction decay to enhance signal-to-noise.

2.3. Molecular modeling

Modeling was performed using Schrodinger’s Maestro Suite (V. 2014-1) [24] with previously described methods [25]. Briefly, the peptide-bound CXCR4-CVX15 crystal structure (pdb 3OE0) [26] was prepared using the Protein Preparation Wizard with hydrogen bonds optimized. A docking grid was generated using Glide [27] centered on the Arg2 – Nal3 residues on the CVX15 peptide. Low-energy conformers of TIQ-10 were generated using MacroModel [28], and these conformers were docked flexibly into the grid. The most favorable pose is presented and discussed.

3. Results and discussion

Given the unique structural information available from the STD NMR experiment, we were interested in using it to probe the binding of small molecule antagonists to the CXCR4 receptor. Although high-level expression of the CXCR4 receptor has been achieved, no bacteria-based system expressing active CXCR4 (including cell-free systems) has been generated [29]. Active CXCR4 was obtained from a constructed CXCR4-expressing CHO cell line that displays sensitivity to the CXCR4 chemokine ligand agonist (SDF-1α) in a cAMP GloSensor Assay (Fig. S1). However, live cells rapidly precipitate in liquid phase NMR tubes resulting in poor quality spectra. To overcome this problem, STDD NMR experiments were performed with membrane extracts obtained from CHO cells expressing CXCR4. To minimize complication from overlapping resonances of the sample milieu, the aromatic signals from the ligand were used in this study to provide accurate NMR integrations.

TIQ-10 (Fig. 1) was selected as an NMR probe compound. This agent is composed of three structural features: 1) a top tetrahydrodroquinoline (THQ) ring, 2) a butyl amine chain, and 3) a bottom tetrahydroisoquinoline (TIQ) ring. The TIQ-10 aromatic 1H NMR signals were assigned using coupling constant networks and chemical shift prediction (Fig. 2A, Fig. S5). H1 is nearest the THQ nitrogen on the TIQ ring and appears at 8.33 ppm. The other two protons from this ring (H2 and H3) present signals at 7.22 and 7.56 ppm, respectively. H4 is located on the bottom TIQ ring near the aniline nitrogen with H2–H4 rounding out the bottom benzyl moiety. H7 and H5 appear at 6.91 and 6.94 ppm, respectively, while H6 and H4 appear at 6.64 and 6.60 ppm, respectively.

The STD spectrum of TIQ-10 in the presence of the same CXCR4-expressing CHO membranes reveals well-resolved and intense signals for each aromatic proton. The STD build-up experiment, wherein the saturation time is varied, reveals that the intensities of the TIQ-10 signals increase with longer saturation times (Fig. 2B and Fig. S3). The longest saturation time (3 s) yields the greatest intensity STD signals. It follows that these signals are the result of specific interactions with CXCR4 in addition to non-specific interactions with other components of the extracts. To demonstrate that the STD enhancements are due to binding similar to the parent...
compound, an identical sample was prepared by incorporating TIQ-15. Addition of the parent compound (TIQ-15) reduces the TIQ-10 STD signals by approximately 50%, namely those from the TIQ benzyl ring (Fig. S2). This result confirms competitive displacement at similar binding sites for the two ligands, but spectral overlap requires additional STD experiments to correct for off-target binding.

To assess the specific interaction between TIQ-10 and CXCR4, an identical sample was prepared with the addition of AMD3100 (a potent and specific CXCR4 antagonist [22]). AMD3100 displaces the weaker binding ligand reflected by lower intensity STD signals for TIQ-10 over all saturation times (Fig. 2C). Comparison of the 3 s saturation time spectra shows that the H4 signal decreased the most upon addition of AMD3100 by approximately 35%. Modest decreases in signal intensity are observed for H1 and H7 (20–30%), while the signals for H2, H3, H5, and H6 did not decrease significantly upon addition of AMD3100 (<10%) (Fig. S3). These spectral changes indicate competitive binding between the two antagonists for CXCR4. The remaining signal from the TIQ-10/AMD3100 spectra (Fig. 2C) are likely the result of off-target interactions with the sample milieu, and this is supported by observation of similar TIQ-10 STD signals observed from samples prepared with un-transfected CHO membranes (lacking CXCR4) (Fig. S2).

To obtain the CXCR4-specific binding epitope, the integration for each proton from the AMD3100/TIQ-10 STD spectrum (Figs. 2C and 3 seconds) was subtracted from the TIQ-10 STD integration (Figs. 2B and 3 seconds) to obtain the STDD enhancements (Table 1). Normalized STDD intensities typically indicate variations in ligand–protein contacts, but these variations are sensitive to different proton relaxation rates [30]. All TIQ-10 aromatic protons exhibit similar T1 values in the presence of CXCR4-expressing membranes (0.45–0.62 s, Table 1) measured by inversion recovery. With an acquisition time of 1 s and a recycle delay of 3 s, T1 does not affect the signal intensities, which indicates that the STDD integrations reflect the true TIQ-10 – CXCR4 binding epitope.

The TIQ benzyl H4 proton exhibits the greatest STDD enhancement submitting that the TIQ nitrogen interacts with the receptor placing H4 in close contact with the protein (Fig. 3A). H7 also displays an intense STDD enhancement indicating that the TIQ ring is flanked by the protein. H5 and H6 display essentially zero STDD intensities advocating that the bottom portion of the TIQ benzyl ring orient towards an opening within the receptor. The second greatest STDD enhancement occurs for H1 ortho-to the THQ nitrogen. This suggests that the THQ nitrogen engages in an interaction with the CXCR4 receptor causing a concomitant close contact with the protein. H2 and H3 STDD enhancements are approximately equal but considerably less intense than H1 proposing solvent exposure at these positions.

Evaluation of the protein–ligand contacts suggested by STDD epitope mapping was achieved by modeling TIQ-10 into the extracellular binding pocket of CXCR4. Crystal structures of the receptor bound to the small molecule IT1t (pdb id 3ODU) and peptide CVX15 (pdb id 3OE0) antagonists reveal structural features of the extracellular binding pocket. The receptor is composed of seven transmembrane helices numbered sequentially from the N-terminus, and the spacious extracellular ligand binding pocket is divided into sub-pockets [26]. The minor sub-pocket is shallow and formed by helices I–III and VII, while the deeper major sub-pocket is formed by helices VI–VII containing the homodimerization interface at helix VI–V [31]. A large extra-cellular loop 2 (ECL2) extends outwards between helices IV and V as a β-sheet capping the pocket. Since STDD demonstrates that AMD3100 and TIQ-10 compete for the same binding pocket, TIQ-10 was docked into the antagonist pocket. Several poses were considered by docking into the IT1t and CVX15 crystal structures and evaluated for agreement with the STDD enhancements. The pose in highest agreement with the NMR data emerged from the CVX15 crystal structure. This pose, depicted in Fig. 3B, predicts a hydrogen bond between the TIQ ring nitrogen and Glu288 and an electrostatic interaction between the butyl amine and Asp171 (Fig. S4 provides detailed images of the complex). In this pose, the THQ ring is placed in the major sub-
Within 25% error of those observed experimentally (Fig. S6). This is the largest calculated STD value ($j_{\text{max}}$). The calculated STDD amplifications for protons within 5.0 Å (i) are measured. The STD amplification factors for H1 and H6 close contact with helix VII and ECL2, respectively. H5 and H6 face helices I and II and sit in the open minor binding pocket. Interatomic distances between TIQ-10 and CXCR4 compiled in Table S1 (Supporting Information) are consistent with the STDD enhancements, the best docked pose and the architecture of the modeled binding site (Fig. 3A–C, respectively). The structural features of this bound model can be used to calculate theoretical STDD enhancements for a proposed bound complex with the CORCEMA method [32]. However, this approach requires values that are challenging to measure accurately for membrane-embedded proteins (correlation time, binding kinetics, and protein concentration). Seeger et al. propose a simple estimate of STDD enhancements that typically falls within 25% error of the experimental values [30]:

$$\text{STD}_{j} = \sum_{i} \frac{1}{P_{i}} \times \%\text{STD}_{j} = \frac{\text{STD}_{j}}{\text{STD}_{j_{\text{max}}}}$$

For each ligand proton (j), the distances (r) to all receptor hydrogens within 5.0 Å (i) are measured. The STD amplification factor (%STD) is then normalized for each ligand proton (j) with respect to the largest calculated STD value ($j_{\text{max}}$). The calculated STDD amplification factors for H1–H3 along the top THQ ring all fall within 25% error of those observed experimentally (Fig. S6). This indicates that the model captures an accurate position of the THQ ring. The model also provides low calculated STDD amplification factors for H5 and H6 consistent with the measurements. However, the model provides lower-than-error calculated amplification for H4 and H7 flanking the bottom benzyl TIQ ring (Fig. S6). This disagreement may be due to membrane interaction within the binding pocket unaccounted for in the model, dynamical effects, or populations of slightly higher-energy binding poses. Nonetheless, the model correctly captures the STDD enhancements for five out of the seven signals. It is worth noting that no other model re-captures more than two STD enhancements within the 25% error threshold (Fig. S7). Thus, for most interactions suggested by the experiment, the modeled pose is consistent with the STDD binding epitope.

The STDD enhancements and structural model offer a rationale for the 1000-fold gain in potency for TIQ-15 over TIQ-10. Firstly, the TIQ-15 nitrogen is protonatable (not amine) stiffening the interaction with Glu288. Secondly, the right-shifted lower aromatic ring allows more favorable hydrophobic interactions within the minor binding pocket. STDD and modeling data thus identify key points of interaction to drive future antagonist optimization. The receptor encompasses the THQ pyridyl ring highlighting a potentially critical role for the nitrogen. The TIQ benzyl ring is flanked by hydrophobic contacts within the receptor positoning a favorable role for this moiety. Lastly, the bottom of the TIQ ring (H5 and H6) does not form close contacts with the protein suggesting available space for synthetic exploitation in future molecular design efforts.

4. Summary

Here, we employed STDD NMR as a tool to determine structural information for a ligand binding to an embedded receptor in membrane extracts. Such information supports a binding pose obtained by molecular modeling and is consistent with rationalizing the difference in activity between TIQ-10 and TIQ-15. These studies and methods are amendable to other membrane proteins and ligands of interest, provided that there is rapid exchange between the bound and free forms. The approaches presented here have potentially broad application in chemical biology, molecular biology and structure-based molecular design.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.08.084.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.08.084.

References


TABLE 1

Parameters of TIQ-10 aromatic protons.

<table>
<thead>
<tr>
<th>Proton#</th>
<th>1H chemical shift (ppm)</th>
<th>1H T2 (sec)</th>
<th>Percent STD enhancement&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Exp.</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.32</td>
<td>0.63 ± 0.04</td>
<td>76</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.22</td>
<td>0.54 ± 0.04</td>
<td>30</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.56</td>
<td>0.58 ± 0.04</td>
<td>34</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.60</td>
<td>0.46 ± 0.05</td>
<td>100</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.94</td>
<td>0.54 ± 0.03</td>
<td>14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6.64</td>
<td>0.48 ± 0.05</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6.91</td>
<td>0.48 ± 0.06</td>
<td>68</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> T2 in the presence of membrane extracts measured via inversion recovery.

<sup>b</sup> STD intensities have an error of ±10%.


