

Tipping the balance: a biased nanobody antagonist of CCR3 with potential for the treatment of eosinophilic inflammation

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The recruitment of eosinophils in both homeostasis and allergy is to a large extent dependent upon the eotaxin family of chemokines. CCL11/eotaxin-1 was the first chemokine discovered to have high selectivity for eosinophils by the use of a guinea pig model of allergic airway disease¹. Following the cloning of mouse and human eotaxin orthologues, CCR3 was identified as the eotaxin-1 receptor, which is expressed at high levels on the surface of eosinophils². Two other eotaxins, CCL24/eotaxin-2 and CCL26/eotaxin-3, were discovered, both showing high selectivity for CCR3. CCR3 is a rather promiscuous receptor, binding almost half of the CC chemokine family, which exhibit varying selectivity and potency as chemoattractants. Consequently, CCR3 has become a magnet for novel therapeutic strategies aimed at targeting the trafficking of eosinophils in disease, for example in eosinophilic asthma and oesophagitis. One hurdle in drug development has been the limited homology between human and mouse CCR3 which has compromised the testing of some potent human CCR3 antagonists in mouse models of allergic disease³.

In this issue of JACI, Grozdanovic and colleagues describe the generation and characterisation of a peptide-based CCR3 antagonist, R321⁴. The peptide has sequence identity with the regions of the second transmembrane (TM) helix and first extracellular loop of both human and mouse CCR3. Targeting G protein-coupled receptors (GPCRs) in such a fashion is not a new idea, indeed, peptide antagonists of GPCRs based on TM helices were reported in the literature as far back as 1999⁵. However, a novel formulation was employed by the authors in which polyethylene glycol was conjugated to the C-terminus, thereby limiting peptide aggregation and

resulting in R321 assembling into nanospheres in aqueous conditions. Assays of CCR3 activation in both human eosinophils and human CCR3 transfectants show that R321 has little if any agonist activity, but selectively antagonises G protein activation when cells are treated with CCR3 ligands. In mouse models of acute allergic airways disease using a combination of allergens, R321 at a dose of 12mg/kg *i.v.* shows excellent efficacy when used both prophylactically and therapeutically. This dose compares favourably with other CCR3 antagonists which have often suffered with problems of bioavailability ³. As the authors state, it will be important to determine the efficacy of R321 in chronic models of allergic disease. Likewise, comprehensive pharmacokinetics are required to assess the half-life of R321 and any inherent toxicity.

Notably, R321 has no inhibitory activity in assays of ligand-mediated CCR3 endocytosis, unlike well-characterized small molecule CCR3 antagonists such as UCB35625 ⁶. Such bias is unusual for a CCR3 antagonist although perhaps not entirely unexpected, since there have been numerous reports that *pertussis* toxin treatment (which uncouples GPCRs from G α i proteins) has little or no effect on chemokine receptor endocytosis, suggesting the two pathways are clearly divergent. The authors suggest that the inability of R321 to inhibit CCR3 endocytosis means that eosinophils do not become tolerized to the compound as CCR3 is not trapped on the cell surface. Instead, CCR3 is internalized and is likely degraded, to be replaced by *de novo* synthesized CCR3 ⁷. Interestingly, R321 has little activity alone in inducing CCR3 endocytosis. This is an important observation, since some therapies rely upon driving GPCR endocytosis rendering cells unresponsive to ligand, for example the treatment of prostate cancer by the Gonadotropin-releasing hormone receptor agonist buserelin.

So how does R321 antagonise CCR3 at the molecular level? Activation of CCR3 is thought to conform to a multi-step model in which CCL11 is tethered predominantly by interactions with the N-terminus of CCR3. This serves to orientate the chemokine such that the chemokine N-terminus can interact with the TM helices of the receptor, breaking several intrahelical interactions and ultimately stabilizing a receptor conformation that activates G protein and recruits arrestin. Notably, CCR3 shares considerable homology with CCR1 (64%) which also undergoes G protein-independent endocytosis and has been shown by Gilliland and coworkers to exist as a homodimer, pre-coupled to both the G α i protein required for signaling and β -arrestin required for endocytosis ⁸. We may therefore surmise that CCR3 also exists as a homodimer (Figure 1A). By mimicking 13 amino acids of the upper portion of TM helix II, we can postulate that R321 breaks interhelical interactions within CCR3 that are required for the activation of G proteins, but not for β -arrestin-dependent endocytosis (Figure 1B). Grozdanovic *et al* showed that CCL11 is able to bind to CCR3 occupied by R321 which is in keeping with the predominant role of the CCR3 N-terminus in binding CCL11. Similarly, Sabroe and colleagues showed that inhibitory concentrations of UCB32625 which binds within the CCR3 TM helices, were unable to displace ¹²⁵I-CCL11 from CCR3 transfectants ⁶.

Gilliland *et al* postulated that drugs that blocked G protein activation but were permissive for endocytosis might form a new pharmacological axis for drug development. In the case of R321 we would appear to have a prototypic drug that does just that. Given the fact that 15 out of the 23 residues in TM helix II of CCR3 are conserved in CCR1, then it plausible that R321 is also an effective inhibitor of

CCR1 which should be examined forthwith. Small molecule antagonists with specificity for both CCR1 and CCR3 are not uncommon and might also be beneficial, given that hyperresponsiveness of eosinophils to the CCR1 ligand CCL3 has been reported in donors with a history of atopy-associated diseases ⁹. Moreover, since the results of a previous clinical trial targeting CCR3 alone were without efficacy in reducing circulating and sputum eosinophil levels, dual targeting of two or more ligand:receptor axes by R321 might provide the long-awaited breakthrough in this area ¹⁰. We eagerly await the progress of this molecule.

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Figure legends

Figure 1. A schematic depicting how R321 could potentially allow ligand-driven endocytosis, but prohibit G protein activation. Panel A. CCR3 exists as a homodimer which can bind a single molecule of CCL11. Binding induces conformational changes in both CCR3 subunits which activates both G proteins and β -arrestin leading to intracellular signalling and endocytosis. Panel B shows the effects of R321 (yellow). CCL11 can still bind and drive β -arrestin-mediated endocytosis of the complex but is unable to induce the conformational changes in the neighbouring CCR3 molecule required for G protein signalling.

The diagram illustrates two pathways for the internalization of the CCL11 receptor, a G-protein-coupled receptor (GPCR). The receptor is shown as a transmembrane protein with seven transmembrane helices (I-VII) and an extracellular N-terminus. The intracellular C-terminus is labeled with the residue R321.

Pathway A (Left): This pathway involves the activation of Gαi. The receptor is shown in an active state, with the Gαi protein (purple oval) bound to the intracellular C-terminus. The Gαi protein is labeled with the residue R321. The receptor is then internalized via endocytosis, as indicated by the downward arrow. The resulting internalized receptor is shown in a vesicle, with the Gαi protein bound to the intracellular C-terminus.

Pathway B (Right): This pathway involves endocytosis mediated by arrestin. The receptor is shown in an active state, with the arrestin protein (yellow oval) bound to the intracellular C-terminus. The arrestin protein is labeled with the residue R321. The receptor is then internalized via endocytosis, as indicated by the downward arrow. The resulting internalized receptor is shown in a vesicle, with the arrestin protein bound to the intracellular C-terminus.

- **Endocytosis**