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IDENTIFICATION AND CHARACTERISATION OF AN INHIBITOR OF INTERLEUKIN-8: A RECEPTOR BASED APPROACH.

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Abstract:
Identification of the extracellular domains of the interleukin-8 (IL-8) receptor led to the synthesis of several peptide sequences. A peptide derived from a single subdomain was shown to inhibit IL-8 binding and to act as a functional antagonist. Ala-scan and truncation strategies identified key areas of this peptide for further work. Copyright © 1996 Elsevier Science Ltd

Interleukin-8 (IL-8) is a pro-inflammatory polypeptide chemokine produced by a number of cell types (e.g., T-lymphocytes, monocytes, endothelial cells, epithelial cells and neutrophils) in response to a variety of stimuli e.g., LPS, IL-1 and TNF.[1] The best characterised of the actions of IL-8 are chemotaxis and activation of the neutrophil.[2] Raised levels of IL-8 have been detected in several disease states[3] and experiments using monoclonal antibodies have indicated that inhibition of the action of this chemokine in some animal models can lead to beneficial effects.[4] IL-8 has been characterised structurally using NMR and X-ray crystallography.[5] These studies indicate that IL-8 is a non-covalently linked homodimer, each monomer consisting of 72 amino acids. IL-8 is believed to exert its actions as a monomer[6] through cell surface receptors. Two receptors for IL-8 on the human neutrophil, IL-8RA[7] and IL-8RB[8], have been cloned and expressed and their deduced amino acid sequences are typical of the class known as G-Protein Coupled Receptors (GPCRs). This class of receptor is characterised by seven transmembrane spanning alpha-helices linking four extracellular and four intracellular domains.

The identification of specific inhibitors of pharmacologically active agents is central to the success of drug-discovery programs. A number of strategies currently exist to achieve this goal. The "High Throughput Screening Approach" relies on assaying the effects of large numbers of compounds to give a low molecular weight lead. The success of this method relies upon the speed of screening, the size of library / libraries available and on the chance of a suitable lead being present in one of the libraries. This type of approach has been particularly successful for finding inhibitors of GPCRs.[9] An alternative method involves using the

available structural information on the targets to design compounds that mimic one of the components and thereby inhibit the interaction. Such an approach has also been successful within the GPCR field, but only by using the structures of small molecule ligands.[10] In the case of the IL-8/IL-8R interaction, the problem is complicated by the size of the ligand (72 residues, MW 8382Da). Some important residues of IL-8 involved in binding to the receptor[11] have been identified, but this has so far failed to lead to low molecular weight inhibitors. An alternative, however, is to mimic the receptor rather than the ligand. This approach might lead to effective compounds provided that the concentration of ligand is not excessive in the physiological situation.

In general it is believed that small molecule agonist/antagonists of GPCRs bind between the membrane associated helices of the receptors.[12] On the basis of the model proposed for C5a, a similarly sized cytokine that also exerts its action through a GPCR, [13] the extended N-terminus of IL-8 might be expected to interact with the transmembrane helices of the receptor. While it is known that residues in this area (Glu-4, Leu-5, Arg-6 and Ile-10) are important for receptor binding,[11,14,15] they are not sufficient to explain *all* the receptor binding. An additional area of residues on the surface of IL-8 has recently been identified as important for receptor binding.[16] Interaction with this area is likely to involve the extracellular domains of the receptor.

We wished to investigate whether peptide fragments identified from the extracellular regions of IL-8 receptors would be effective in inhibiting the IL-8 response. In addition we wanted to evaluate how such peptides might be modified in order to provide useful leads for drug design programmes. The availability of high quality structural data on IL-8 suggested that it might be possible to interpret interactions of complexes in terms of molecular structure information. This information is the heart of the "biostructural approach" to lead optimisation.[17]

Analyses of the primary sequence of the IL-8RA (the receptor most selective for IL-8) via hydropathicity plots[18] suggested sequence segments likely to be the seven membrane spanning helices. This in turn allowed the identification of putative extracellular and intracellular loops.[19] (Figure 1.)

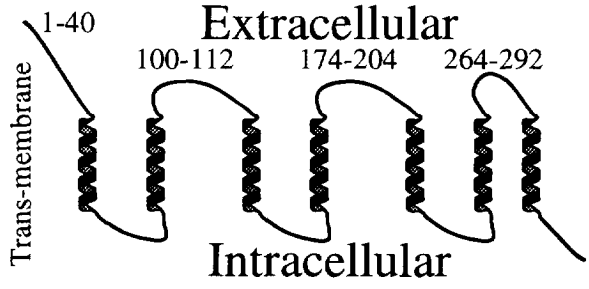


Figure 1. Cartoon of typical GPCR showing residue numbers of extracellular domains for IL-8RA.

Peptide fragments corresponding to extracellular sequences were synthesised using solid-phase techniques. These were analysed for their ability to inhibit the binding of I^{125}-IL-8 to neutrophils and, in some cases, the generation of intracellular calcium transients by IL-8.[20]

Table 1: K_is for receptor fragments from the extracellular domains (ECDs).*

Domain	residue number	K_i†	Sequence
1st ECD	(1-19)	>30mM	Ac-MSNITDPQMWDFDDLNFTG-NH_2
1st ECD	(20-40)	0.3mM	Ac-MPPADEDYSPXMLETETLNKY-NH_2
2nd ECD	(100-112)	>30mM	Ac-VNGWIFGTFLXKV-NH_2
3rd ECD	(174-185)	10mM	Ac-FRQAYHPNNSSP-NH_2
3rd ECD	(186-204)	>1mM	Ac-VXYEVLGNDTAKWRMVLRI-NH_2
4th ECD	(264-292)	>1mM	Ac-ADTLMRTQVIQETXERRNNIGRALDATEI-NH_2

X = (S)-2-aminobutyric acid.

* Peptides were synthesised using a Milligen 9050 peptide synthesiser under continuous flow conditions employing Fmoc chemistry on PepsynK resin with AM linker. They were synthesised as N-terminal acetyl and C-terminal amides to prevent unwanted charged interactions at the termini. Resin cleavage conditions were TFA:phenol:EDT:anisole (95:2.5:2.5:2.5) 2h, RT. The resin was filtered and the solvent removed. The peptide was precipitated in ether, filtered and dried. Purification was by RP-HPLC on an Aquapore octyl column (20micron, 100mmx10mm).The elution gradient comprised 95%A-95%B over 15min where A=0.1%TFA in water and B=0.085%TFA in 70%acetonitrile. The peptides were checked for purity by analytical RP-HPLC and structures were validated by mass spectrometry, ^1H NMR and amino acid analysis.

† K_is were obtained in an IL-8 ligand binding assay. Neutrophil membranes were prepared by sonication of the cells in a buffered protease inhibitor cocktail and isolated by centrifugation. Membranes (approx. 2µg protein) were incubated for 90 min at 4 °C in 100µl of phosphate buffered saline containing 1% BSA, 0.1% sodium azide, 0.25nM ^{125}I-labelled IL-8 and inhibitor. The assay was carried out in a 96 well microtitre plate with 0.22micron pore size filters. Free and bound ligand were separated by rapid filtration with washing. The amount of radioactive material bound to the filters was determined with a gamma counter and the results corrected for non-specific binding. A computerised curve-fit programme (EBDA) was used to estimate K_i values.

The results indicated that a sequence from the N-terminus of the receptor had the greatest inhibitory effect (1st ECD 20-40). Attempts to simplify this sequence led to a 10 aminoacid sequence with reduced but still measurable activity, Ac-MPPADEDYSP-NH_2, K_i 1.7mM (1). It was possible to demonstrate by NMR[21] that this compound bound to IL-8 at a concentration close to its K_i, providing evidence for its proposed mechanism of action. Identifying regions of importance in this peptide was problematic since any significant reduction in activity, attributable to the loss of an interaction with the ligand, would provide compounds with activities beyond the limit of detection. Extending the peptide to include further C-terminal residues did not result in increased activity. However expanding the peptide to include N-terminal residues led to a significant increase in bioactivity, Ac-MWDFDDLNFTGMPPADEDYSP-NH_2 K_i 13µM.[22]. This peptide was also shown by NMR[21] to bind IL-8 at a concentration similar to its K_i and was a functional antagonist[20] of the action of IL-8 (IC_{50} 19µM). The peptide was also highly selective for IL-8 over C5a.

We were interested in determining whether the activity of the peptide could be localised to a shorter sequence to give a smaller peptide lead. Alternatively, if important residues were scattered through the sequence, we intended to identify those residues that were acting merely as "scaffolding" and which could be replaced to lead to simpler structures.

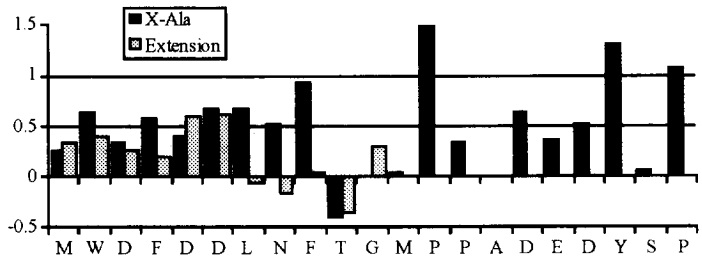
The so-called "ala-scan" is a method frequently used to identify key residues.[23] In this method non-alanine, non-glycine residues are altered to alanine to determine a role for the sidechains beyond the β-carbon. This method has an advantage over other mutation strategies in that it enables the importance of residues to be quantified; i.e. presuming that conformational changes are minimal, alterations in activity can be interpreted as due to loss of binding interactions rather than gains in detrimental interactions. The "ala-scan" methodology has been used to good effect in proteins.[24] However, in conformationally mobile systems, such as peptides, the interpretation of the results can be more difficult since changes in activity must be partitioned between binding interactions and changes in the manifold of low energy conformations occupied. The results of the "ala-scan" of the lead peptide are shown in Table 2. Pro-13, Tyr-19 and Pro-21 (entries 13, 18 and 21) were identified as being important for the activity of the 21mer with a change in activity of >10 fold. However, all three of these residues were present in the much less potent 10mer (1); thus some feature other than the side chains of the N-terminus was presumably responsible for the greater potency of the longer peptides. One possible reason would be the existence of an interaction between IL-8 and the peptide backbone or side chain up to the β-carbon. A series of peptides of increasing length was therefore also synthesised (Table 2). Figure 2 shows, in graphical form, the result of adding each new residue and compares this with the effect of mutating the same residue to alanine. Additions of single amino acids failed to give any large increases in activity. Instead, increasing the length of the peptide from a 15-mer to a 21-mer (entries 26-32) merely gave a 3-5 fold increase in potency as each N-terminal residue was added. Since a corresponding small change in activity is also observed in the "ala-scan" (entries 2-7), this could arise from the presence of weak interactions between the peptide sidechain in this region and IL-8. This contrasts with the results obtained for the middle of the peptide (entries 8-10 and 23-25), e.g., Phe-9 was identified by Ala replacement as relatively important (entry 10), but adding it as an N-terminal residue gave no significant increase in activity (entry 24). One explanation of this is that the central section of the peptide is required for individual residues elsewhere to be presented in the correct binding conformation.

Thus we have identified a fragment of the IL-8 receptor which is a potent inhibitor of the IL-8/IL-8R interaction, and have identified both some key residues in this peptide and a region which appears to act as "scaffolding." Hence considerable simplification of the peptide may be possible by replacing this "scaffolding region" with an appropriate linker. Further work to investigate this hypothesis is underway.

Table 2. Activities of peptides

Entry		Peptide sequence		K_i
1.	Ac	MWDFDDLNFTGMPPADEDYSP	NH_2	13μM
2.	Ac	**A**WDFDDLNFTGMPPADEDYSP	NH_2	23μM
3.	Ac	M**A**DFDDLNFTGMPPADEDYSP	NH_2	55μM
4.	Ac	MW**A**FDDLNFTGMPPADEDYSP	NH_2	28μM
5.	Ac	MWD**A**DDLNFTGMPPADEDYSP	NH_2	49μM
6.	Ac	MWDF**A**DLNFTGMPPADEDYSP	NH_2	32μM
7.	Ac	MWDFD**A**LNFTGMPPADEDYSP	NH_2	62μM
8.	Ac	MWDFDD**A**NFTGMPPADEDYSP	NH_2	61μM
9.	Ac	MWDFDDL**A**FTGMPPADEDYSP	NH_2	42μM
10.	Ac	MWDFDDLN**A**TGMPPADEDYSP	NH_2	107μM
11.	Ac	MWDFDDLNF**A**GMPPADEDYSP	NH_2	5μM
12.	Ac	MWDFDDLNFTG**A**PPADEDYSP	NH_2	14μM
13.	Ac	MWDFDDLNFTGM**A**PADEDYSP	NH_2	400μM
14.	Ac	MWDFDDLNFTGMP**A**ADEDYSP	NH_2	27μM
15.	Ac	MWDFDDLNFTGMPP**A**EDYSP	NH_2	55μM
16.	Ac	MWDFDDLNFTGMPPAD**A**DYSP	NH_2	29μM
17.	Ac	MWDFDDLNFTGMPPADE**A**YSP	NH_2	42μM
18.	Ac	MWDFDDLNFTGMPPADED**A**SP	NH_2	265μM
19.	Ac	MWDFDDLNFTGMPPADEDY**A**P	NH_2	15μM
20.	Ac	MWDFDDLNFTGMPPADEDYS**A**	NH_2	155μM
21.	Ac	MPPADEDYSP	NH_2	1700μM
22.	Ac	GMPPADEDYSP	NH_2	890μM
23.	Ac	TGMPPADEDYSP	NH_2	2050μM
24.	Ac	FTGMPPADEDYSP	NH_2	1850μM
25.	Ac	NFTGMPPADEDYSP	NH_2	2700μM
26.	Ac	LNFTGMPPADEDYSP	NH_2	3100μM
27.	Ac	DLNFTGMPPADEDYSP	NH_2	750μM
28.	Ac	DDLNFTGMPPADEDYSP	NH_2	190μM
29.	Ac	FDDLNFTGMPPADEDYSP	NH_2	120μM
30.	Ac	DFDDLNFTGMPPADEDYSP	NH_2	68μM
31.	Ac	WDFDDLNFTGMPPADEDYSP	NH_2	27μM
32.	Ac	MWDFDDLNFTGMPPADEDYSP	NH_2	13μM

Figure 2. Graphical summary of Ala scan/extension results:



Ala scan: log (K_i ala scan peptide/ K_i parent 21mer)
Extension: log (K_i n residues/K_i n+1 residues)

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