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CHEMOATTRACTANT RECEPTORS IN DRUG DISCOVERY
FPR2 AND CCR2 - TWO POTENTIAL TARGETS

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ABSTRACT

Chemoattractants and chemoattractant receptors have many important functions in multicellular organisms, not least for their role in regulating migration of leukocytes. The receptors are also involved in many pathologies and they have, since they were cloned in the 1990ies, been regarded as attractive targets for development of drugs against cancer, viral infections, and inflammatory/autoimmune diseases.

Although chemotactic molecules are very heterogeneous in terms of chemical structure most of them mediate their actions through binding to a G-protein-coupled receptor (GPCR). The formyl peptide receptors (FPRs) are chemoattractant receptors expressed on phagocytes and have important implications in host defense and inflammation as well as in resolution of inflammation. One of the goals was to identify FPR2 specific ligands with similar anti-inflammatory and proresolution properties as the eicosanoid lipoxin A4 previously described. A small compound library was screened for both agonist and antagonists and selected compounds were analyzed with respect to anti-inflammatory properties such as inhibition of NADPH oxidase activity and degranulation in primary neutrophils. Receptor selectivity was investigated by compound stimulation and desensitization studies in presence of receptor-specific tools. While the agonist screen resulted in non selective hit compounds with proinflammatory properties, the antagonist screen led to identification of anti-inflammatory agents with different selectivity.

Another chemoattractant receptor binds CCL2, a member of the superfamily of chemokines. The function of CCL2 and its receptor (CCR2) is of importance for monocyte recruitment, and together this receptor ligand pair has many implications in inflammation. Accordingly, the importance of the CCL2/CCR2 axis in the recruitment of inflammatory cells and fibroblasts into fibrotic tissues was hypothesized as a mechanism for induction of fibrosis. There is, however, emerging evidence indicating that CCL2 may promote fibrosis also by other mechanisms such as activation of the fibroblast. The myofibroblast, synthesizing large amounts of extracellular matrix components (ECM) in response to profibrotic cytokines and growth factors, is the key cell in fibrosis. The effects of CCL2 on human fibroblasts with respect to ECM expression were studied and it was found that CCL2 might have a more multifaceted role in fibroblast activation than previously described.

The truncated form of CCL2, CCL2 (1,9-76), also known as 7ND, is a receptor antagonist and is described to have therapeutic effects in several animal disease models such as bleomycin-induced lung fibrosis. The interaction between the 7ND and the human as well as the murine CCR2 was studied *in vitro* and data showed that 7ND was an inhibitor of human CCR2 but a very low affinity binder of the murine receptor. 7ND was, however, shown to downregulate fibrotic markers in murine fibroblasts, but in accordance with its inability to bind CCR2 the downregulation was found to be independent of this receptor.

LIST OF PUBLICATIONS

I. Huamei Forsman, **Christina Kalderén**, Anna Nordin, Erik Nordling, Annika Jenmalm Jensen, Claes Dahlgren
Stable formyl peptide receptor agonists that activate the neutrophil NADPH-oxidase identified through screening of a compound library
Biochemical Pharmacology 81 (2011) 402–411

II. Hulya Cevik-Aras, **Christina Kalderén**, Annika Jenmalm Jensen, Tudor Oprea, Claes Dahlgren, Huamei Forsman
A non-peptide receptor inhibitor with selectivity for one of the neutrophil formyl peptide receptors, FPR1
Biochemical Pharmacology 83 (2012) 1655–1662

III. **Christina Kalderén**, Margareta Forsgren, Ulla Karlström, Karin Stefansson, Robert Svensson, Magnus M. Berglund, Gunnar Palm, Martin Selander, Maj Sundbom, Joakim Nilsson, Annelie Sjögren, Kristina Zachrisson, Stefan Svensson Gelius
A truncated analogue of CCL2 mediates anti-fibrotic effects on murine fibroblasts independently of CCR2
Biochemical Pharmacology 83 (2012) 644–652

IV. **Christina Kalderén**, Charlotte Stadler, Margareta Forsgren, Mona Sydow-Bäckman, Stefan Svensson Gelius
CCL2 mediates antifibrotic effects in human fibroblasts independently of CCR2
Manuscript

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LIST OF ABBREVIATIONS

α-SMA	α-Smooth muscle actin
AA	Arachidonic acid
ATL	Aspirin-triggered lipoxin
C5a	Complement component 5
cAMP	Cyclic adenosine monophosphate
CCL2	Chemokine (C-C motif) ligand 2
CCR	C-C motif receptor
CHO	Chinese hamster ovary cells
CIA	Collagen-induced arthritis
COX-1	Cyclooxygenase-1
CTGF	Connective tissue growth factor
CXCR	C-X-C chemokine receptor
ECM	Extracellular matrix protein
EMT	Epithelial mesenchymal transition
FITC	Fluorescein isothiocyanate
FLIPR	Fluorometric imaging plate reader
fMLF	N-Formyl-L-methionyl-L-leucyl-L-phenylalanine
FPR	Formyl peptide receptors
GAG	Glycoseaminoglykans
GPCR	Guanine nucleotide – binding protein - coupled receptors
HEK293	Human embryonic kidney cells
HFL-1	Human fetal lung fibroblast
HTS	High-throughput screening
IKKβ	Inhibitor of nuclear factor-κB kinase subunit
IL-13	Interleukin-13
IPF	Idiopathic pulmonary fibrosis
LXA4	Lipoxin A4
LPS	Lipopolysaccharide
Mab	Monoclonal Antibodies
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex molecules
MMP	Matrix metalloproteinases
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate

oxidase	
NF-kB	Nuclear factor κ-light-chain-enhancer of activated B cells
PDGF	Platelet derived growth factor
PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PIP2	Phosphatidyl 4,5-biphosphate
PLA_2	PhospholipaseA_2
PMN	Polymorphonuclear neutrophil
QuinC1	Quinazinolione
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SSc	Systemic scleroderma
TGFβ	Transforming growth factor β
TIMPs	Tissue inhibitors of metalloproteinases
TM	Transmembrane helix
TNFα	Tumor necrosis factorα
UIP	Usual interstitial pneumonia
TM	Transmembrane helix

1 INTRODUCTION

The immune system is vital to defend us from microorganisms and contribute to tissue injury repair. In response to foreign invaders as well as trauma, inflammatory responses are orchestered by a large number of different inflammatory cells recruited to the site of inflammation where they kill microorganisms and contribute to wound repair.

Recruitment of inflammatory cells is mediated by chemottractants. They are important signaling molecules of different sizes and structures that integrate important functions in inflammation. Chemoattractants bind to chemoattractant receptors on immune cells and induce migration and other crucial functions of the inflammatory cells.

Dysregulations of the inflammatory response and tissue repair may lead to chronic inflammation and autoinflammatory disease as well as fibroproliferative disorders. Traditionally, inflammatory diseases are treated with NSAIDs and glucocorticosteroids, and the latest contribution is the large group of antibodies and antibody-like drugs such as the TNFα-inhibitors. However, several inflammatory disorders, together with fibrotic diseases still constitute a large medical need of new drugs.

With their important roles in inflammatory responses, chemoattractant and their receptors are interesting targets for development of new drugs. By modulation of their responses, the goal is to slow down harmful immune reactions and prevent chronic inflammation. The first drug candidates are in progress and hopefully we will see new such anti-inflammmatory drugs on the market within the next few years.

In this thesis different strategies to modulate the responses of chemoattractant receptors, together with associated opportunities and challenges, are discussed. Experiences will be discussed based on research of two chemoattractant receptors; identification and characterization of small molecule modulators of the FPR receptors and characterization of a previously described peptide antagonist of the CCR2 receptor for anti-inflammatory/antifibrotic therapeutic use.

1.1 Immune reactions and host defense

We are constantly exposed to different types of potentially harmful microorganisms and as a part of our daily life we need to defend ourselves from such attacks. We also sometimes run into slight or serious injury and these threats to our health are handled in our body by reactions aiming to heal and repair.

The innate immune system is the first line of our defense against microorganisms and many parts of the system were developed long before adaptive immunity. Innate immunity is universal, acting within minutes upon stimulation. In brief the innate immune system relies on three foundations, i) recognition of pathogens, ii) ability to

kill these pathogens and, iii) sparing the host tissues or being self-tolerant. The innate immune apparatus is built up by cellular as well as humoral components and these two parts can be attributed to recognition and/or effector functions that interact in a complex network [1].

The major cellular components are patrolling professional phagocytes of myeloid origin that seek invading microbes and when these are recognized they should be engulfed and killed. Among these patrolling cells are macrophages which are distributed throughout the body and they are always ready for action. Macrophages also have supervising and organizing roles in both innate and adaptive immunity. By elaboration of chemoattractants they also recruit other myeloid cells expressing chemoattractant receptors to the site of infection. The neutrophil granulocyte is one such cell, a specialized killer endowed with a broad array of weapons and of prime importance in innate immunity [1].

Phagocytes are equipped with receptor structures that react to all kinds of threats, so-called danger signals that are recognized by surface and endosomal receptors. Toll-like receptors (TLRs) [2, 3] and formyl peptide receptors (FPRs) [4] are examples of such recognition structures. Microbial derived pathogen-associated molecular patterns (PAMP), and damage- associated molecular patterns (DAMP) [5] as well as cytosolic or nuclear proteins are examples of signaling molecules. When these are host derived, they are normally not exposed but released through trauma.

Recognition of a danger signal initiates a cell based effector cascade involving among other potential tissue destructing molecules such as reactive oxygen species. These are produced by an enzyme complex termed the phagocyte NADPH oxidase [6]. Autocoid substances such as histamine and bradykinin that cause local vasodilatation and cytokines such as interleukin-1 and TNFα that stimulate the endothelial lining of the vessels to upregulate adhesion molecules, are also parts of the cascade. Mobilization of adhesion molecules results in the slowing down of circulating leukocytes and progressively increased adhesion of cells to the endothelial cell layer facilitating the next recruitment step, migration from blood into the inflamed tissue [7].

Some antigens are not neutralized by the innate immune system but will be taken up by highly specified antigen-presenting cells (APC), dendritic cells or macrophages and transported via the lymph to the lymph nodes. In the lymph nodes the antigen will be presented in complex with major histocompatibility complex molecules (MHC) on the surface of APC to naïve T-cells. Activated APC upregulate co-stimulatory molecules that synergize with naïve T-cells and contribute to the activation of the naïve T-cell. The T-cell has become an armed effector T-cell acting in specific against cells carrying the antigen.

The activated T-cell initiates the synthesis of interleukin-2 which promotes the proliferation and differentiation of the activated T cell. The activation may give rise to different subtypes of T-cells, cytotoxic CD8, CD4 Th1 and CD4 Th2 T-cells depending on which antigen is presented to the naïve T cell [8]. Cytotoxic CD8 T cells have the capability to directly kill target cells carrying the antigen while CD4 cells act through activation of macrophages by interferonγ or by activation of B-cells. B-cells can also be

activated directly by microbial antigens. The activated B-cells proliferate and differentiate to plasma cells secreting antibodies specific to the triggering antigen.

Activation of T and B- cells is part of the adaptive immune system. This system when compared to the innate immune system is a more recently evolved immune response that is found only in vertebrates and is considered to be overlapping with the innate immune system. In reality the two systems are highly integrated and support each other.

The humoral parts of the innate immune system include many different molecules in extracellular compartments. Sensing components are for example acute-phase proteins such as the mannose-binding protein, the lipopolysaccharide binding protein (LBP) and the C-reactive protein. These are synthesized in the liver upon cytokine stimulation and bind either directly to microbes or to constituents with a bacterial origin. Elaboration of acute-phase proteins has systemic effects and is one component of the inflammation reaction that causes fever. Other effector molecules of importance for the humoral innate immune system include complement components, lysozyme, lactoferrin and antimicrobial proteins/peptides acting by destroying bacterial cell walls or biological membranes [9].

In addition to cytokines, prostanoids and leukotrienes are important proinflammatory mediators. Phospholipase A2 cleaves phospholipids in the cell membrane and arachidonic acid (AA) is formed and released. This constitutes the backbone of prostanoids synthesized by the enzymes cyclooxygenase-1 and 2 (COX-1, COX-2) or leukotrienes formed by lipooxygenase (5-LOX). The subclass of prostanoids includes the prostaglandins, thromboxanes and prostacyclins. These lipid mediators, acting via both autocrine and paracrine mechanisms have several proinflammatory effects. These include inducing vasodilatation of blood vessels, cell migration and cell damage causing typical reactions of inflammation, fever, redness and pain [10].

Resolution of inflammation is the process leading to homeostasis and restored tissue and this will be further discussed in chapter 3.2.

1.2 Inflammation and fibrosis-two pathologies related to dysregulations of the immune response

Inflammation

The hallmark of invading microbes or injury is an inflammatory reaction aiming to heal and repair. Acute events are immediate and lead to local swelling, redness, pain, heat and sometimes dysfunction of the affected organs. These manifestations are caused by increased blood flow, relaxing effects on blood vessels, release of proinflammatory mediators, extravasation of fluids from blood and lymph vessels into the infested tissue and influx of proinflammatory cells. Normally the inflamed tissue/organ is healed after a couple of days but in exceptional cases the inflammation is extended. The

inflammation may be provoked by persistence of an infectious organism, a nonliving irritant material or an abnormality in the host's response to its own tissue.

The exact mechanisms and causes of autoimmunity/autoinflammation is an emerging field and much research still remains to be carried out before we understand the complexity and how the immune system sometimes fails, with disease instead of protection as the final outcome. When the immune system recognizes a "self molecule", the response results in the development of autoreactive effector cells or formation of autoreactive antibodies, a scenario that might develop into a chronic autoimmune disease [11]. Emerging evidence shows that cells traditionally associated with the innate as well as the adaptive immune systems may be involved in the development of autoimmunity. The chronic inflamed tissue is characterized by infiltration of aggressive inflammatory effector cells together with increased levels of proinflammatory mediators that inevitably cause tissue damage.
Autoimmune diseases can be systemic such as in Sjögrens syndrome and rheumatoid arthritis (RA) or affect local tissues as for example in diabetes and Crohn´s disease. Inflammatory diseases include many common and severe disorders, such as RA, multiple sclerosis (MS), psoriasis and inflammatory bowel disease (IBD).

RA is a chronic inflammatory disease affecting the synovial tissue in joints of hands and feet. The inflammation is characterized by infiltration of inflammatory cells into the joints leading to swelling, proliferation of synoviocytes and pain. Infiltrating cells such as macrophages secrete proinflammatory factors such as TNFα, which is indirectly involved in the destruction of cartilage and bone resulting in disability [12, 13].

MS is a chronic disease of the central nervous system. In similarity with many other inflammatory diseases, it is characterized by infiltration of inflammatory immune cells. Infiltration of T-cells and macrophages in the brain causes destruction of myelin sheets of neurons and apoptosis of myelin forming cells (oligodendrocytes). Neurons are affected resulting in impairment of conduction of signals leading to physical and cognitive disabilities [12, 14].

Psoriasis is an inflammatory skin disease characterized by red, scaly and raised plaques. Psoriasis is caused by a chronic cutaneous pathologic process that is driven by the interactions between inflammatory cells (T cells, dendritic cells, macrophages and neutrophils) and keratinocytes (cells of the epidermis). The disease is induced by infections, stress, antigenic stimuli or a secondary effect to various medications. The skin converts to a psoriatic lesion associated with thickening of the skin and increased amounts of inflammatory cells are found in the dermis [12, 14, 15].

IBD is the generic name of chronic inflammatory conditions of the gastro intestinal tract. The most common diseases are Crohns´s disease and ulcerative colitis. The clinical pathology is similar but these diseases can be distinguished by different locations, immunological and histological patterns. However both diseases are postulated to be T-cell dependent, and TNFα, released from inflammatory cells is responsible for proinflammatory signaling [12, 16, 17].

Fibrosis

Fibrotic disorders are thought to have many different possible etiologies. Fibrosis is the end result of chronic inflammation reactions induced by different stimuli such as pathogenic organisms, autoimmune reactions mediated through B-cell produced auto-antibodies, allergens or tissue components released through injury. However, in many cases the original cause of fibrotic disease remains unknown. Interestingly, there is accumulating evidence that the mechanisms driving fibrogenesis are distinct from those regulating inflammation [18-20].

Tissue repair (Figure 1) is a fundamental process that allows replacement of dead or damaged cells with new cells of the same type. But sometimes the process is dysregulated and normal tissue is replaced by connective tissue. The healing process becomes pathogenic and may result in formation of a permanent scarring of the affected tissue that secondarily leads to organ failure and death. Sustained production of growth factors such as platelet derived growth factor (PDGF), connective tissue growth factor (CTGF) and transforming growth factor (TGFβ), fibroblast growth factor (FGF), angiogenic factors (VEGF) and fibrogenic cytokines (IL-13, IL-4) and components of the renin-angiotensin-aldosterone system, mainly angiotensin II, stimulate vascular remodeling and excess deposition of extracellular cellular matrix components (ECM) such as collagen and fibronectin (Figure 1)[19, 21].

The key mediator of fibrosis is the activated fibroblast (the myofibroblast) over-producing extracellular matrix proteins that accumulate in the fibrotic tissue. The myofibroblast phenotype may be distinguished from resident fibroblasts through its over expression of α-smooth muscle actin (α-SMA) and collagen type I. The myofibroblast may differentiate from cells of different origin; resident tissue fibroblasts, by epithelial mesenchymal transition (EMT) [22, 23] and circulating bone-marrow derived fibrocytes being examples [24]. Collagen turnover and ECM remodeling is regulated mainly by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Dysregulation of the balance between these may result in accumulation of collagen over time [21].

Anti-inflammatory drugs such as corticosteroids or immunosuppressive agents are in some cases able to relieve the symptoms but there is no drug that actually cures serious fibroproliferative disorders such as systemic scleroderma (SSc) or idiopathic pulmonary fibrosis (IPF).

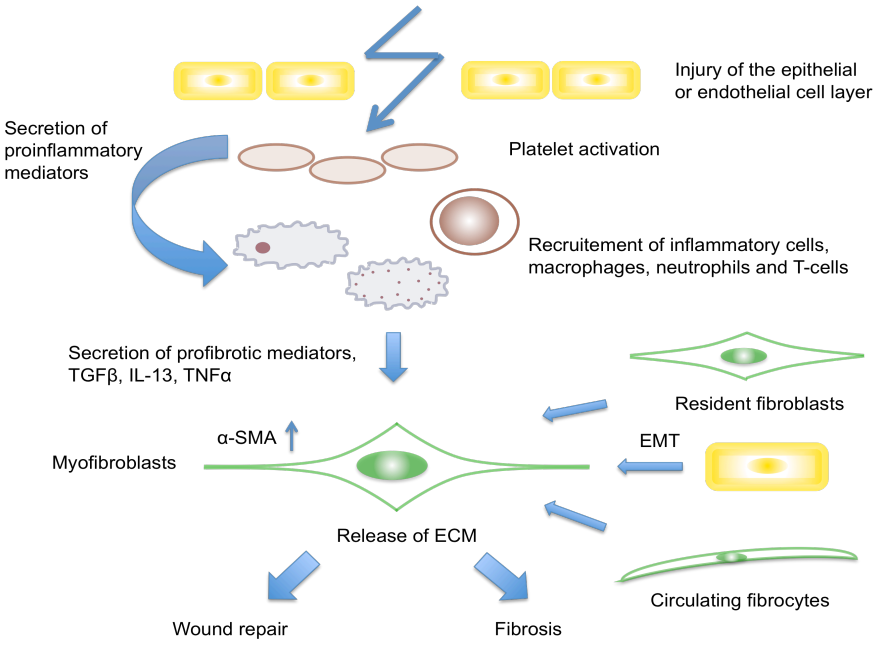


Figure 1. Dysregulation of wound repair may lead to fibrosis.
Wound healing: when epithelial or endothelial injury occurs, inflammatory mediators are released leading to an antifibrinolytic coagulation cascade triggering platelet activation and blood clot activation. Produced growth factors, cytokines and chemokines stimulate recruitment and proliferation of leukocytes. Leukocytes, such as macrophages and neutrophils contribute to eliminating tissue debris and invading organisms and production of cytokines and chemokines that are necessary for epithelial and endothelial cells that migrate to the centre of the wound. Lymphocytes and other cells secrete profibrotic cytokines such as PDGF, IL-13 and TGFβ leading to proliferation and differentiation of fibroblasts of different origin, or via epithelial/mesenchymal transition (EMT) into myofibroblasts. In the resolution phase myofibroblasts release extracellular matrix proteins and promote wound contraction. Epithelial/endothelial cells migrating over the basal layers regenerating the damaged tissue finalize the wound healing process [18].

1.3 Established anti-inflammatory therapies

Traditional NSAIDs and COX-2 inhibitors

The most frequently used anti-inflammatory drugs are the NSAIDs (non-steroidal anti-inflammatory drugs), and both aspirin and ibuprofen belong to this group. NSAIDs are used to treat acute and chronic pain and they are used also against chronic inflammatory disorders such as osteoarthritis. The therapeutic effects of these drugs include decreased vasodilation, oedema, pain and fever, and their mechanism of action is through inhibition of two enzymes, COX-1 and COX-2. This inhibition results in reduced formation of prostaglandins.

NSAIDs also exhibit other important therapeutic effects, as they affect the synthesis of thromboxane, resulting in attenuated platelet aggregation. This property is double-edged, on the one hand a benefit is that stroke and myocardial infarction is prevented, but on the other hand the risk for unintended bleeding may be increased. Accordingly, adverse advents are gastric bleeding following long-term use of COX1-inhibitors. In rare cases also the hepatic and renal functions are affected.

In order to overcome the adverse advent of gastric bleeding, efforts have been made to develop selective COX-2 inhibitors. Unfortunately these were found to exhibit other more serious adverse effects such as stroke and heart attack caused by slightly increased levels of thromboxane. As a consequence, in 2004 Vioxx was taken out of the market [25]. At present Celebrex is still prescribed to patients with RA and osteoarthritis.

Glucocorticosteroids

Glucocorticoids (GCs) are broadly used as therapeutic agents in various inflammatory disorders including conditions such as asthma, RA, eczema and sepsis. GCs are derivatives of the natural endogenous anti-inflammatory steroid-hormone cortisol. The members of the glucocorticoid family, exemplified by dexamethasone and prednisolone, bind to the two glucocorticoid receptors (a and b) and mediate a spectrum of anti-inflammatory effects on cells expressing these receptors. The effects include inhibition of synthesis of cytokines, inflammatory mediators such as prostaglandins, transcriptional activity of COX-2, chemokines, complement factors and receptors of all these factors.

The benefits of GCs are short term dampening of inflammation, vascular changes and limited immunosuppression but use is also associated with serious side effects at long term treatment; hypertension, thrombosis, changes in behavior and memory, gastrointestinal bleeding, osteoporosis and effect on renal function [26].

Biologics

The most recent contribution to the field of anti-inflammatory therapies is the use of monoclonal antibodies and antibody-like fusion proteins. Monoclonals against TNFα (infliximab) bind the cytokine with high affinity and this binding prevents TNFα from binding to its receptor. Infliximab has revolutionized the treatment of patients suffering from RA, Crohn´s disease, ulcerative colitis and psoriasis. However, there are serious side effects of infliximab including increased risk of tuberculosis and other infections and worsening of preexisting heart disease. Etanercept, another type of TNF blocker, is a fusion protein consisting of the soluble human TNFα 2 receptor fused to the Fc fragment of human IgG1. Etanercept has the function of a decoy receptor, eliminating TNFα from the circulation. It is used in treatment of RA and psoriasis and preliminary data shows that Etanercept is associated with less side effects than TNFα monoclonal antibodies [27].

Following the appearance of infliximab-based therapy, a number of other monoclonals have been introduced and these have effects on other inflammatory conditions. These include antibodies recognizing CD11a (efalizumab) as treatment of psoriasis and natalizumab recognizing the integrin $\alpha_4\beta_7$ as treatment of MS and Crohn´s disease. Antibodies against the B-cell epitope, CD20 (rituximab) and CTLA4–immunoglobulin fusion protein (abatacept) that disrupts dendritic cell–T cell interactions, are other antibody or antibody like molecules shown to be useful for treatment of patients with RA. Today there are more than 30 different therapeutic monoclonal antibodies and fusion proteins that have been approved around the world of which the 15 best selling are shown in Table 1. Another 250 are in clinical trials focusing on treatment of malignancies, autoimmune and inflammatory diseases and infectious diseases [28-30].

TABLE 1. The 15 best selling therapeutic monoclonal antibodies and fusion proteins with indications anti-inflammatory and autoimmune disease (www.pipelinereview.com April 2012). Rec-recombinant, RA-rheumatoid arthritis, TNFα-tumor necrosis factorα, IL-interleukin, RR MS-relapsing remittent multiple sclerosis, CTLA4-cytotoxic T lymphocyte antigen 4, CAPS- Cryopyrin-associated periodic syndromes, LFA3-ECD-Leukocyte function-associated antigen-1-extracellular domain

Product Name	Target	Class of Compound	Indication
adalimumab	TNFα	Rec fully human mab	RA
etanercept	TNFα	Dimeric fusion protein of soluble TNF-R + CH2 and CH3 domains of Fc of Ig	RA
infliximab	TNFα	Rec chimeric IgG1 mab	RA
golimumab	TNFα	Rec fully human IgG1 mab, once monthly SC	RA; psoriatic arthritis;
certolizumab pegol	TNFα	PEGylated rec humanized Fab' fragment	Crohn's , RA
natalizumab	α4/ß1/7 integrin	Rec humanized IgG4 mab	RR MS
omalizumab;	IgE	Rec humanized mab	Severe allergic asthma
abatacept	B7-1 (CD80) and B7-2 (CD86)	Rec fusion protein of CTLA4+ Fc of IgG1 (hinge, CH2 + CH3)	RA and juvenile idiopathic arthritis
eculizumab	Complement C5	Rec humanized (hybrid IgG2/4) full mab	PNH
tocilizumab	IL-6R	Rec humanized mab	RA
ustekinumab	IL-12 (p40) and IL-23	Rec fully human IgG1 full mab (UltiMAb)	Psoriasis
canakinumab	IL-1 beta	Rec fully human IgG1 mab	CAPS, incl. Muckle Wells Syndrome
rilonacept	IL-1	Trap (2 IL receptor domains + CH2 +CH3 of Fc)	CAPS
belatacept	B7-1 (CD80) and B7-2 (CD86)	(A29Y + L104E) point-mutated rec fusion protein of CTLA4+Ig	Kidney transplantation
alefacept	CD2	Rec dimeric fusion protein of LFA3-ECD and CH2 and CH3 domains of Fc of IgG1 for injection	Psoriasis

Traditional DMARDs

DMARDs (disease-modifying antirheumatic drugs) are small synthetic compounds that not only reduce the symptoms of the disease but also actually slow down the progression and prevent tissue damage. The term DMARDs is derived from the first indication of e.g. methotrexate for RA. The drug has today various indications such as psoriasis [31] and systemic lupus erythematosus (SLE) [32].

1.4 New strategies to identify anti-inflammatory and antifibrotic agents

New anti-inflammatory targets

As mentioned, considerable progress has been achieved in the treatment of inflammatory diseases during recent years. However, despite success, many patients and diseases are unresponsive to these new drugs and long-term treatment may also result in development of resistance to the drug. In addition, some of these new drugs have occasionally had deleterious effects in that the sensitivity to severe infections has increased. Other drawbacks with the new biopharmaceuticals are the sometimes very high costs and that they cannot be delivered orally. Thus, there is a need for new strategies to treat chronic and autoinflammatory diseases and there is a need for additional targets in the search for anti-inflammatory drugs [30].

A new generation of targets emerged during the 1990s was the cytokine superfamily, including TNFα, IL-1, interferons, growth factors of lymphocytes (IL-6, IL-2) and chemokines promoting activation, maturation and differentiation of cells involved in immunity and inflammation. Another group of targets are the toll-like receptors (TLRs) sensing microbial products or products of inflamed tissue. Downstream of these target molecules, several components of the signaling pathways such as transcription factors (NF-κB), kinases (p38, JNK, JAK/STAT) have been evaluated as new targets for development of anti-inflammatory drugs [33].
The NF-κB system of transcription factors regulates a large number of cytokines, chemokines, adhesion molecules and COX-2. Amongst these important activators of inflammation are the TNFα receptor family, the IL-1/TLR receptor family, the Nod-like receptor family (NLR), IL-17 family and B and T cell receptors. A large number of regulating proteins are involved in regulation of the NF-κB pathway and the pharmaceutical industry are developing modulators targeting many of them.

p38 kinase is known for stabilizing the mRNA of AU repeats occurring in inflammation-related genes including those coding for TNF and COX2. A few p38 modulators are being evaluated as an anti-inflammatory treatment in clinical trials.

The JAK/STAT signaling pathway downstream hematopoietic cytokine receptors and interferons are involved in inflammatory disease. A JAK3 inhibitor, tofacitinib, has been developed and found to be efficacious in clinical trials for RA. It has recently been recommended for approval by the FDA arthritis advisory committee [34].

Moreover, immunosuppressors inhibiting the pathways leading to T-lymphocyte activation, such as IL-2, are interesting as targets. New calcineurin inhibitors prevent IL-2 production via deactivation of NFAT (nuclear factor of activated T cells). They have been approved for atopic dermatitis and have shown promising results in a clinical trial of psoriasis [35].

Another interesting T-cell-targeted therapy is FTY720, a synthetic drug targeting the sphingosine 1 phosphate receptor on T-cells, preventing egress of lymphocytes from the thymus and secondary lymphoid tissues is now approved for relapsing MS [36]. In addition, rapamycin analogues are being evaluated in clinical trial for RA and MS. They target the kinase mTOR in lymphocytes leading to inhibition of proliferation and IL-2 production [37].

Furthermore, IL-17 is a proinflammatory cytokine implicated in inflammatory disease. It is produced by certain subsets of T helper cells such as Th17, and mediates production of several proinflammatory cytokines and chemokines, e.g. IL-8, CCL2, IL-1 and TNF. Brodalumab, an anti IL-17 receptor antibody has significantly improved plaque psoriasis in a phase II clinical study [38].

New antifibrotic targets

Another therapeutic area with a large unmet medical need is the group of severe fibroproliferative diseases. Several growth factors, cytokines, lipids, and peptides are associated with fibrosis; TGFβ, tyrosine kinases (cAbl), CTGF, endothelin-1 (ET-1), interleukins such as IL-13 and IL-6, lysophosphatidic acid receptor 1 (LPA1) and Wnt.

Accordingly various potential target groups are in evaluation for treatment of fibrotic disease, including i) antibodies against IL-13 and CTGF, a co-factor of TGFβ, are being evaluated in clinical trials (both in phase II) and results are awaited with interest, ii) an antibody against TGFβ as well as inhibitors of a tyrosine kinase (cAbl) down-stream of TGFβ (imatinib) have so far shown lack of efficacy: iii) LPA1 is defined as a profibrotic target and clinical trials phase II will soon be initiated, iv) several existing T-cell targeted therapies are evaluated for the indication of fibrosis: v) rapamycin and an antibody against the IL-2 receptor (basiliximab), vi) a fusion protein (abatacept) blocking of the co-stimulatory molecules B7 on antigen-presenting cells, and vii) two antibodies targeting B-cells, (rituximab and belimumab) may also be interesting to evaluate as antifibrotic therapies.

Another group of potential targets are transcription factors such as STAT4 which is downstream of interferonγ and IL-2, T-bet, a regulator of the Th1 immune response, and AP-1 which is downstream of TGFβ and PDGF.

Moreover, several intracellular molecules may be interesting targets in the search for antifibrotic agents. Peroxisome proliferator-activated receptor gamma (PPARγ) is one example described to be down-regulated in biopsies obtained from scleroderma patients. Rosiglitazone, a PPARγ agonist, is already used as an insulin-desensitizing therapy in diabetes mellitus, which encourages evaluation as antifibrotic therapy in humans. Other examples are S100A4, an intracellular molecule signaling downstream TGFβ, and Notch, which are activated in leisonal skin of SSc patients.

Finally, 5-hydroxytryptamine (5-HT, serotonin) and its receptor $5HT_{2b}$ are interesting antifibrotic targets. 5-HT is released upon activation of platelets due to microvascular injury. 5-HT levels are increased in SSc patients and might be a link between vascular injury and fibrosis. 5-HT induces the synthesis of extracellular matrix proteins in dermal fibroblasts via the $5HT_{2b}$ receptor and $5HT_{2b}$ inhibitors prevented onset of experimental fibrosis and ameliorated established fibrosis. Results from a first small study in humans are awaited [39].

2 STRATEGIC AND CONCEPTUAL THINKING IN TARGETING CHEMOATTRACTANT RECEPTORS

Another group of molecules that were explored as possible targets in late 1990s are the chemoattractants, mediators of cell recruitment in inflammation. Evidence linking chemoattractants and their receptors to different pathological states has been drawn from knockout and transgenic animal models, genetic association studies and expression studies in human disease tissue as well as experimental approaches with agonists and antagonists [40-44]. Beside their implications in infections and malignancies, they play important roles in inflammatory and autoimmune disorders such as atherosclerosis, RA, psoriasis, IBD as well as fibroproliferative diseases and many attempts are being made to modulate them in order to develop new drugs [12, 43, 45-49].

2.1 Basic biology of chemoattractants and their receptors

Chemoattractants and their receptors

Cell migration is a key event of several physiological processes in multicellular organisms. It is a central process in embryonic development, wound healing, and immune surveillance, but also in many pathologies such as inflammation, fibrosis and malignancy. The driving force of migration is external signals and cells are moving directionally, guided by concentration gradients of such specific mediators, chemoattractants. Accordingly, the process is called chemotaxis [50, 51].

As a response to inflammatory stimuli, leukocytes migrate from blood through the endothelial cell layer to sites of inflammation. Migration is induced in response to concentration gradients of classical chemoattractants secreted from resident macrophages, destructed tissue or foreign invaders. The group of chemoattractant inflammatory mediators is heterogeneous with respect to chemical structure; the natural chemoattractants are proteins, peptides, phospholipids or lipids. N-formylated peptides (i.e., fMLF), defensins, C3a and C5a (fragments from the complement factors C3 and C5), leukotriene B4, platelet-activating factor (PAF) and in addition the large chemokine superfamily all belong to the group of classical chemoattractants [52]. However, several other endogenously expressed mediators e.g. cytokines and growth factors are attributed to have chemoattractant properties. In addition, recent research has led to the emergence of a new class of synthetic small molecule chemoattractants [53].

With few exceptions chemoattractants induce cell migration through activation of receptors belonging to a family of structurally related so called seven transmembrane and Guanine nucleotide – binding Protein - Coupled Receptors (GPCR) expressed on the cell surface of leukocytes.

Chemoattractant receptors belong to the rhodopsin-like class A GPCR family. As mentioned these receptors have substantial similarities in that they form seven transmembrane helices (TM 1-7), enriched in hydrophobic amino acids (aa) and they all have an extracellular N-terminal and an intracellular C-terminal. They also have three intracellular (il1-3) and three extracellular loops (el 1-3) containing more hydrophilic amino acids. A disulfide bond linking cysteine residues in extracellular loops 1 and 2 is also common. The TM3 region contains a preserved DRY (aspartate, arginine, tyrosine) motif and an aspartate residue in TM2, and these have been shown to be of importance for signaling from the activated receptor [40].
There are no signature residues or motifs common to all chemoattractant receptors that distinguish them from other types of GPCRs. However, some consensus features characterize them and distinguish them from other receptors of the GPCR family. They are shorter (approximately 350 aa) than other GPCRs, largely due to a very small i3 loop and a relatively short C-terminus. The short (16-22 aa) i3 loop is enriched in basic aa [54, 55] and the N-terminus is in most cases acidic [53, 56, 57].

Ligand binding induces conformation changes of the receptor catalyzing the switch from the GDP-state to the GTP-state of the G-protein resulting in dissociation of its α subunit from the $\beta\gamma$ subunits and these transduce several cellular events. Activation of chemoattractant G-protein coupled receptors are in most cases coupled via one of the subgroups of the Gα subunits namely the Gαi, characterized by sensitivity to pertussis toxin [52]. Upon activation the receptor-expressing cell starts to move in the direction of the ligand source (Figure 2).

Furthermore, chemoattractants contribute to the proinflammatory response via their receptors in a number of other ways. Ligand activated receptors induce important physiological processes such as production of reactive oxygen species (released to destroy/eliminate foreign microbes), secretion of proinflammatory mediators, gene transcription, receptor mobilization, and stimulation of cell adherence [52]. Moreover, emerging evidence suggests that chemoattractants are critical also for the immunomodulatory properties of the adaptive immune response. Accumulating evidence suggests that chemokines affect dendritic cell maturation, macrophage activation, B cell antibody class switching, and T cell activation [44, 58].

An additional attribute of chemoattractant receptors is that their signaling may mediate pro as well as anti-inflammatory effects (dual signaling). The chemoattractant receptor of interest may be expressed on both inflammatory and inflammatory-modulating cells. This is exemplified by the opposite effects of the on one hand CCR2 expressing inflammatory monocytes and on the other hand CCR2 expressing immuno down–modulating regulatory T-cells in a collagen-induced arthritis model [59].

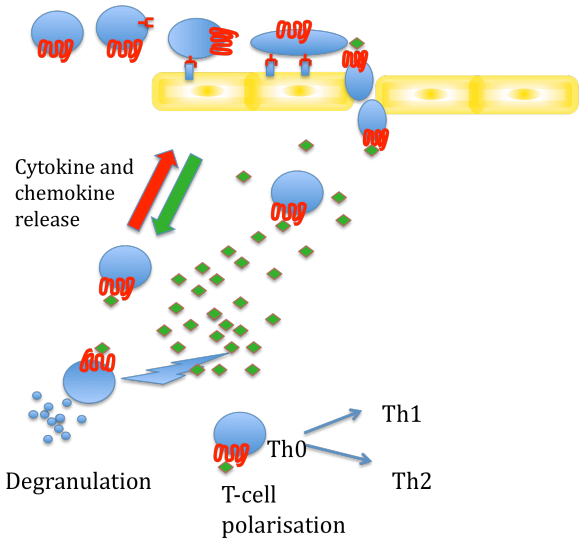


Figure 2. Leukocytes expressing chemoattractant receptors transmigrate through the endothelial cell layer of the vessel wall in the direction of increasing concentrations of chemoattractants released from a site of inflammation. Ligand activated receptors induce important physiological processes such as degranulation/production of reactive oxygen species, secretion of proinflammatory mediators and T-cell polarization.

In accordance with other GPCRs, chemoattractant receptors are phosphorylated and desensitized upon agonist binding and this process leads to an activation of the downstream intracellular signaling cascade required for receptor internalization and dampening of response of ligand binding. In addition, ligand induced activation of the receptor may also result in cross-desensitization of other receptors [60]. Desentitization of the receptor at high ligand concentration may result in the classic bell-shaped curve [61].

Some chemottractant receptors may be expressed exclusively in subtypes of leukocytes or in local tissues and certain cellular functions, such as recruitment of monocytes may be mediated by more than one receptor [41, 62, 63]. It is also well known that chemoattractant receptors are redundant, each receptor attracts more than one ligand and each ligand may interact with more than one receptor [43, 64].

Chemokines and their receptors

In contrast to other classical chemoattractants, the members of the chemokine family have conserved sequences and are structurally related [53]. The chemokine family includes 50 low molecular weight proteins (7.5-10 kDa, except for the 76 kDa membrane associated fractalkine). They are classified based on number and positions of conserved cysteine residues (C, CC, CXC and CX3C). Despite the fact that sequence identity between chemokines can range between 20 to 90%, their tertiary structures are highly conserved. The structure of chemokines comprises three distinct domains: (1) a highly flexible N-terminal domain, which is constrained by disulfide bonding between the N-terminal cysteine(s); (2) a long loop that leads into three antiparallel β-pleated sheets; and (3) an α-helix that overlies the sheets (Figure 3) [65].

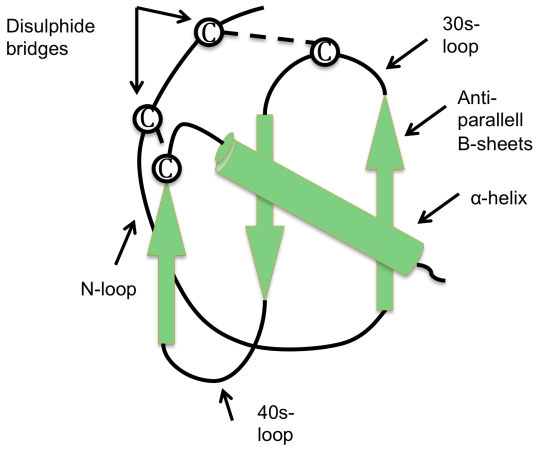


Figure 3. Schematic representation of the three-dimensional structure of a C-C motif chemokine [65].

The chemokines mediate their effects through 20 different receptors within the chemokine superfamily of GPCRs. Chemokine receptors are structurally related and distinguish from other GPCRs in that they have 2 disulfide bonds. Beside the disulfide bond between the first and second extracellular loop there is a second disulfide bond between the N-terminal and the third extracellular loop. The nomenclature of chemokine receptors follows the names of their ligands, C, CC, CXC and CX3C. The receptor family also includes decoy receptors (DARC, D6, CCX-CKR) that take part in chemokine clearance [40].

Furthermore, chemokine receptors can form homo-, hetero- and even oligomeric structures with other structurally related receptors and influence each other's signaling. The different oligomeric structures are controlled by the expression levels of the different receptors and the presence of their ligands [66].

To add further complexity, chemokines do not only bind to receptors on the leukocyte surface but also to glycosaminoglycans (GAG) exposed on endothelial cells and deposited in the extra cellular space. Chemokines oligomerize on the endothelial cell surface to which leukocytes are attracted [66] and in many cases, oligomerization is critical for their proper function *in vivo* [67]. Dimerization, oligomerization and binding to GAGs may also protect chemokines from proteolytic degradation [68].

2.2 Targeting chemoattractants and their receptors in drug discovery

Chemoattractants and their receptors are important regulators of the innate as well as the adaptive immune response and dysregulations may lead to pathologies. These receptor-ligand pairs control and organize trafficking and functional activation of leukocytes in health and disease in processes that are not yet completely understood. Expression and stimulation of chemoattractant receptors is very much dependent on the homeostasis of the tissue and the influence of different pathologies. Although large efforts have been made and different strategies have been validated for targeting chemoattractants and their receptors, rather few drugs have been approved on the

market. In retrospect, targeting chemoattractant receptors has shown to be a rather challenging task with the risk for many pitfalls as the biology of this target group is very complex [41, 42, 69].

Several approaches are used in drug discovery to modulate the effects of chemoattractants and their receptors. They include the development of small molecule agonists/antagonists, modification of peptide ligands, monoclonal antibodies (Mab) and Fab fusion proteins.

Screening for small molecule receptor modulators

One strategy for targeting the chemoattractant receptors is to screen for small compound receptor agonists or antagonists using high-throughput screening (HTS) of small compound chemical libraries. Using this rational large-scale approach many thousands of compounds can be screened resulting in a large spectrum of receptor ligands exhibiting different sizes, receptor affinities, functional responses and biophysical properties such as solubility and bioavailability.

As the primary selected screening hits do normally not fulfill all the requirements of a lead compound, a lead optimization process follows the primary screening, a process that is in many cases time and resource consuming. Large efforts are normally required to develop a new chemical entity and besides efficacy, parameters such as absorption, distribution, metabolism, excretion and toxicity (ADMET) need to be taken into account [70].

On the positive side is the production costs of small molecules which are normally more favorable compared with those for biologics. In addition, small molecule compounds have a greater potential of being orally available than biologics which are often susceptible to proteolytic degradation. An interesting opportunity is that small compounds may be cell permeable and, in similarity with cyclosporine and COX (cyclooxygenase) inhibitors, can act within the cell downstream of the surface receptor [69].

Small molecule high-throughput screening strategies of GPCRs are either based on functional cellular assays or cell-free biochemical assays, such as binding assays. A binding assay can be performed in a very rational way using homogenous membrane preparations of the receptor-expressing cell and a radiolabeled ligand. The downside of this approach is that only ligand affinity is measured meaning that receptor agonists are not distinguished from antagonists or inverse agonists. A binding assay is a good complement in the evaluation of hits, thus indicating if the selected compound actually binds to the receptor or unspecifically to the surface or is taken up by the cell interacting intracellularly [71].

If the screening strategy is based on a functional cellular assay, other considerations need to be taken into account. The conventional way of functionally screening GPCRs is using modification of G-protein dependent second messengers such as calcium release or cAMP accumulation. These two are well established “high-

throughput compatible" read-outs. However, also other general assay concepts have recently been developed such as beta-arrestin based functional assays and whole cell label free assays [71].

Calcium release screening makes it possible to screen thousands of compounds within a short time using high-throughput equipment like a FLIPR (fluorometric imaging plate reader). The signal to noise ratio is large and hit selection is a relatively straightforward process. Traditionally, transfected cell lines such as human embryonic kidney cells (HEK293) or chinese hamster ovary cells (CHO), over-expressing the receptor of interest have been used. If the ligand stimulated transfected cell line does not respond with calcium release, complementary Gα proteins, such as Gα16, may be co-transfected to shift the agonist induced response to calcium release [72]. It is also possible to use transformed or tumor cell lines that endogenously express the protein of interest. One such example is the THP-1 cell line (human acute monocytic leukemia cell line) expressing CCR2B. In addition, the use of primary cells is increasing, as it is a more relevant *in vitro* model.

All the strategies discussed are associated with their specific advantages and disadvantages and one has to be aware of the different interpretations of the responses that can be made. First of all, the relevance of the functional response needs to be considered. The most conventional approaches such as measuring modified calcium release in transfected cell lines may not reflect modifications of the most relevant disease-related cellular event. If it is possible one should consider the use of a method that actually makes it possible to select compounds modulating the functional response of interest [73].

Using primary cells may however be associated with other problems such as low signal to noise ratio. Isolation of primary cells is also resource consuming and the preparations may differ depending on individual status of organ or blood donors or on the animal used. It is thus an advantage to use a limited number of cells/organs both for the primary screen and the hit confirmation.

Biologics

The natural activation of many of the chemoattractant receptors involves the interaction between large macromolar ligand and receptor molecules and simulation of that interaction with molecules of a limited size may be associated with difficulties [69]. It has been proved relatively difficult to identify small molecule blockers/inhibitors of chemoattractant receptors. One reason may be that chemoattractants, in particular chemokines, are large molecules and use other types of binding pockets than other smaller GPCR ligands such as monoamines. In addition, chemokine receptors may have more than one binding pocket as they normally attract more than one type of chemokine. Consequently, it may not be enough with one compound that interacts with only one of the binding sites to efficiently block the receptor [42]. Accordingly, other approaches may be relevant for the development of chemokine receptor antagonists.

A mechanism used by the immune system to regulate and fine-tune the inflammatory reaction and chemokine activity is to modify ligands [74]. Chemokine variants may be formed through polymorphism at the gene level or through post-translational modifications such as proteolytic truncations, glycosylations, deamination and citrullination. CXC- as well as CC-chemokines may also lose activity through proteolytic degradation/truncation [74]. According to this, another approach to target chemoattractant receptors is to generate modified peptide ligands. This chemokine engineering strategy opens many possibilities and one big advantage compared to small molecule library screening is that the starting molecule is a bioactive high-affinity molecule. One example is truncated CCL2 (1,9-76), described in 4.3 that is transformed from a potent CCR2 agonist into an antagonist. However, it is clear from the work performed so far that chemokine/receptor axes have unique properties and no general rules for how to transform an agonist into an antagonist have been established [75-77]. This strategy also has other limitations in that chemokines are relatively large and complex molecules that are sensitive to protelytic degradation and relatively rapidly cleared from the circulation. To overcome this issue, biologics such as small ligand peptides or soluble receptors are fused to a stabilizing partner protein e.g. an antibody-derived Fc fragment.
However, the production and purification of large biomolecules using recombinant technology is complex and expensive unless the size of the molecule can be reduced.

An interesting approach to modify the activity of chemokine receptors is to develop non-GAG binding chemokines. These peptide ligands will not be sequestered via GAG binding at the cell surface but will stay in the circulation for a longer time than conventional chemokines. They will neither contribute to the formation of the solid-phase concentration gradients leading to chemotaxis of leukocytes. These modified chemokines mediate their anti-inflammatory actions by homologous and heterologous desensitization of the receptors [60].

A third alternative to modulate the reactivity is to neutralize either the chemoattractant or the receptor with an antibody. However, few therapeutic antibodies against chemoattractants have yet been approved for clinical use and so far no antibody targeting a GPCR has been approved. A possible reason is that GPCRs are well integrated in the cell membrane and are not exposed enough to trigger a strong immunogenic response [78].

General concerns about targeting chemoattractant receptors

There are general issues in common for all these approaches. Selectivity of the target is a relevant issue in all drug discovery, not least in the development of drugs with effects on chemoattractants or their receptors. The fact that chemoattractant receptors preferably are studied *in vitro* in primary cells expressing multiple chemoattractant receptors, subgroups of sequence and structurally related receptors, may complicate the analysis of selectivity. The use of relevant tools in the form of specific agonist and antagonist is thus essential.

Another important aspect is the cross species activity of the receptor modulator of interest. The compound may be selective for the human receptor, activating e.g. the murine receptor weakly or not at all. Human chemoattractant receptors often have two or more murine orthologues and the identity of the dominating one in relation to the human origin is not always clear. This is clearly illustrated when looking at the FPRs; there are three human FPR receptors whereas the number in mice far exceeds this number [79].
It may also be the other way around. CXCR1 is the main neutrophil chemoattractant receptor in human for recruitment of neutrophils by the cytokine IL-8. The gene coding for CXCR is lacking in mice that instead use CCR1. Consequently, diseases involving neutrophils and macrophages are sometimes difficult to validate in mouse models [62].
This is one of the most challenging issues in development of new drugs and so far there is no ideal solution. One strategy to compensate for this is to perform *ex vivo* studies in human primary cells and the human tissue of interest [62, 69].

Two examples of anti-inflammatory drugs targeting chemoattractant receptors on the way to the market

CCR9 is central for migration of immune cells to the intestine. Its ligand CCL25 is expressed at the mucosal surface of the intestine and is elevated in intestinal inflammation (IBD, Crohn´s disease and ulcerative colitis). A small molecule antagonist (CCX282) has been developed which inhibits CCR9-mediated cell recruitment in a murine colitis model. The compound is being evaluated (phase III) for the indication of Crohn´s disease [80].

One of the chemoattractant axes more extensively studied and explored by the pharmaceutical industry is the **C5-C5a** receptor axis. Targeting the C5-C5a receptor axis is an appropriate example that illustrates most of the different strategies applied in targeting chemoattractants and their receptors for the development of anti-inflammatory drugs. C5 is part of the complement system of the innate immunity and activation leads to cleavage of C5 to C5a and C5b that subsequently form the terminal complement complex (C5b-9). C5a mediates its proinflammatory effects via two GPCR receptors, CD88 and C5L2. CD88, a G-protein coupled receptor, is well established as a central mediator of proinflammtory events in host defense against foreign microorganisms as well as in chronic inflammatory diseases. CD88 is expressed on a wide variety of cells and activation by C5a mediates several proinflammatory cellular events. The C5-C5a receptor axis has been implicated in several large inflammatory disorders such as sepsis, arthritis, irritable bowl disease, ischemia reperfusion and neurodegenerative diseases.
Several attempts have been made to neutralize C5 and C5a with monoclonal antibodies. To date there is one anti-C5 antibody, eculizumab (Alexion Pharmaceuticals), a humanized antibody approved for treatment of paroxysmal nocturnal hemoglobinuria (PNH), a serious hematologic disorder. As eculizumab is a humanized antibody there is a potential risk for immunogenic reactions in patients upon long term treatment and a second generation of fully human anti-C5 antibodies are in development.

Another approach to target the C5-C5a receptor axis is the development of CD88 receptor antagonists. Bearing in mind that the 8 residues of the C-terminal of C5a retain the activity of CD88, truncated high affinity peptide receptor antagonists have been developed. These compounds have demonstrated efficacy in animal models and some of them have been evaluated in clinical trials. Unfortunately so far these trials have been halted for different reasons; lack of efficacy in patients, off-target activities with other receptors, lack of cross species activity or that they were potentially immunogenic.

In addition, several non-peptide small molecule C5a antagonists have been developed. The development of these types of antagonists has been halted for various reasons such as lack of cross-species activities, toxic effects, low efficacy and lack of oral activity. One compound, the orally active CD88 antagonist CCX168, has demonstrated efficacy in a model of anti-neutrophil cytoplasmic autoantibody (ANCA) disease and has now entered clinical trials (phase II). Finally, a series of anti-CD88 antibodies were reported, e.g. Neutrazumab, they are so far in preclinical development [45].

3 THE FPR FAMILY-A GROUP OF MULTIFACETED TARGETS OF ANTI-INFLAMMATORY THERAPEUTIC INTEREST

3.1 Basic biology of FPRs

Unlike other leukocyte chemoattractants, N-formyl peptides could originate from either an endogenous source, such as the mitochondrial proteins of ruptured host cells, or an exogenous source, such as the proteins/peptides of invading pathogens. N-formyl peptides mediate their effects via the formyl peptide receptor family (FPR) of GPCR receptors. The human FPRs constitute three members whereas the family has seven members in mouse [81]. The human family of these receptors was cloned in 1990 [82] and in 2010 the nomenclature of the receptors, FPR1, FPR2 and FPR3, was revived from the previously named FPR, FPRL-1 and FPRL-2 [79]. These three receptors display large sequence homology and accordingly exhibit pronounced cross reactivity with several ligands. Both neutrophils and monocytes/macrophages have the ability to recognize and respond to a large number of molecules that bind FPRs and serve as "danger signals". Emerging evidence shows that agonists and antagonists of these receptors regulate a large number of biological functions [43, 83].

The most extensively studied of the three receptors is FPR1 [5, 84] which is the high affinity receptor of formyl-methionyl-leucyl-phenylalanine (fMLF) and the first neutrophil GPCR to be cloned and sequenced [82]. fMLF was the first structurally defined chemoattractant, a bacterial produced peptide that triggers a variety of proinflammatory biological activities in neutrophils. fMLF induces calcium release, protein kinase cascades and membrane polarization at low nanomolar concentrations, while release of superoxide is more notable at higher nanomolar concentrations [4, 83, 85-87].

Beside fMLF, FPR1 binds with high affinity and is activated by a large range of ligands such as virus-derived peptides e.g. T20/T21 (HIV-1 gp41) [43], the synthetic peptide tool, WKYMVm [88] and non-formylated peptides [83, 89].

Gradients of receptor ligands induce migration of FPR expressing neutrophils from blood into inflamed and infected tissue where they exert antibacterial effector functions such as degranulation and upregulation of integrins. The granules in mature neutrophils contain a variety of proteins and can be classified as peroxidase-positive and peroxidase-negative. The peroxidase-positive azurophilic granules are those carrying anti-microbial proteases and myeloperoxidase (MPO). Upon neutrophil stimulation, azurophilic granules fuse with the phagosome and release their contents thereby exposing the ingested microbe to toxic agents resulting in bacterial death [6]. The enveloping membranes of the peroxidase-negative granules contain a variety of functionally important membrane proteins such as complement receptor 3 (CR3/CD11b), the NADPH oxidase component flavocytochrome b_{558}, and FPR1.

These membranes fuse with secretory vesicles and the plasma membrane and constitute reservoirs of important integral membrane proteins ready to be recruited to the surface of activated neutrophils [90].

Associated with degranulation of stimulated neutrophils is the "phagocyte respiratory burst", a coincidental abrupt increase of oxygen consumption, leading to production of toxic reactive oxygen species (ROS). This is one of the antibacterial strategies used by neutrophils in host defense, and responsible for this cellular event is the activation of the NADPH oxidase enzyme complex. NADPH oxidase is segregated into the membrane and cytosol in resting neutrophils; the heterodimer of gp91^{phox} and p22^{phox}, parts of the flavocytochrome b_{558}, is localized in a membrane while the other components, p47^{phox}, p67^{phox} and p40^{phox}, are found in the cytosol of resting cells. Upon activation p47^{phox}, p67^{phox} and p40^{phox} are translocated to the membrane where the NADPH oxidase is finally assembled. Active NADPH oxidase in the membrane transfers electrons from the NADPH in the cytosol across the extracellular membrane where superoxide anions are produced in the presence of molecular oxygen (Figure 4). Superoxide can also be produced in intracellular compartments where it dismutases to H_2O_2 or reacts with other oxygen species generating a number of different oxidants [6].

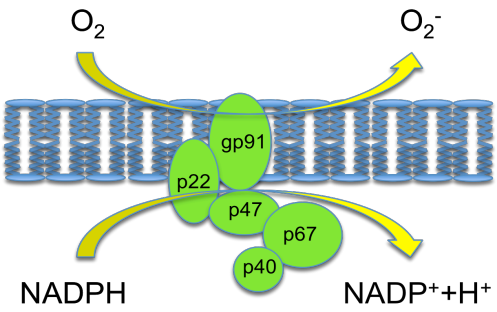


Figure 4. Active NADPH oxidase in the membrane transfers electrons from the NADPH in the cytosol across the extracellular membrane where superoxide anions are produced in the presence of molecular oxygen [91].

Furthermore, FPR signaling has been reported to regulate the survival of infiltrating cells within the site of inflammation. Proinflammatory and prosurvival formylated peptides and endogenous serum amyloid A (SAA) and fMLF compete with pro-apoptotic, proresolution factors such as annexin A1 and LXA4 described in 3.2 [92].

Signaling via FPR1 is regulated by two processes, receptor desensitization and agonist-induced internalization. Desensitization occurs either by direct ligation or following activation of similar GPCRs. After initial activation of FPR1 with fMLF, the receptor rapidly reduces its responses to secondary stimulation with the same agonist (homologous desensitization). FPR1 is also desensitized via ligation of other GPCRs (heterologous desensitization) such as C5aR or CXCL2/IL-8, in a concentration-dependent manner. FPR1 is quite highly placed in the hierarchy of GPCR cross talk, and it can downregulate a variety of GPCRs more efficiently than the other way round. The mechanism of receptor internalization has been shown to be

independent of β-arrestin but is suggested to depend on the conformational differences of each ligand-FPR1 complex [43].

3.2 FPR2 - a chemoattractant receptor with dual functions

The structures of FPR1 and FPR2 are very similar and there is considerable promiscuity across these receptors. However, FPR2 is an unusual receptor as it is activated by a large number of exogenous and endogenous ligands of different chemical structures (peptides, lipids and small molecules), capable of inducing pro as well as anti-inflammatory responses (Figure 6). The signal transduction is therefore complex and far from fully investigated. However, the intracellular parts of FPR1 and FPR2 have close homology in their intracellular domains and accordingly, the proinflammatory cellular responses exhibit large similarities between the two receptors. FPR2 is a low-affinity receptor of fMLF, that together with amyloidogenic peptides (beta-amyloid42 and acute-phase protein SAA), HIV-envelope gp120, the truncated form of the ß-chemokine, CKß 8-1 and the synthetic peptide WKYMVM, induce proinflammatory actions in neutrophils such as NADPH oxidase activation and degranulation. In addition, the interaction between SAA and FPR2 was reported to induce NF-κB activation and IL-8 secretion by neutrophils [93].

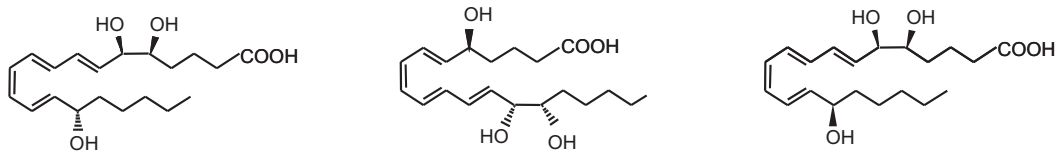
As mentioned, FPR2 is also described to exhibit significant anti-inflammatory and proresolution responses *in vitro* and *in vivo*. These actions were mediated by endogenous human peptides such as annexin A1 and synthetic derived peptides such as Ac2-26 [94], humanin and one of the most extensively studied FPR2 agonists, the eicosanoid lipoxin A4 (LXA4).

Lipoxin A4

LXA4 and LXAB (Figure 5) are structurally and functionally distinct amongst the lipid-derived mediators in that they possess a trihydroxytetraene structure and primarily mediate anti-inflammatory activities [95, 96]. It was shown by displacement of radiolabeled LXA4 that LXA4 binds with high affinity and selectivity to the FPR2 receptor on both primary neutrophils and FPR2-transfected CHO cells [79]. In addition, the two FPR2 ligands, LXA4 and WKYMVM were shown to inhibit the calcium release signal elicited by each other suggesting that they activate the same receptor in neutrophils, FPR2 [97]. However, it is important to recognize that conflicting data has recently been reported, suggesting that LXA4 does not act via FPR2 in neutrophils [98]. Furthermore, LXA4 was also reported to bind to the nuclear aryl hydrocarbon receptor [99] and the leukotriene receptors [100].

Upon proinflammatory stimulation, in particular by PGD2 and PGE2, lipoxins are synthesized from arachidonic acid via cellular and transcellular interactions by two major routes involving leukocyte 5-lipooxygenase and 12-lipooxygenase [101-103]. Aspirin-triggered lipoxin A4, (ATL) (Figure 5), is a stable lipoxin-derivative with similar functions synthesized via an acetylated form of COX-2 [103]. Intake of aspirin

(acetyl-salicylic acid) encourages the acetylation of COX-2 and may be one the therapeutic actions of aspirin.



LXA4 LXB4 ATL

Figure 5. Structures of the eicosanoids LXA4, LXAB and ATL. ATL is the enantiomer of LXA4, synthesized by an acetylated COX-2.

LXA4 and ATL have been reported to exhibit very specific anti-inflammatory properties both *in vitro* and *in vivo*. These effects have been extensively described during the last 15 years [43, 104, 105] and LXA4 and ATL were described as mediators with "multifaceted" anti-inflammatory and proresolving activities. LXA4 and modified stable analogues were described to mediate several cellular events in neutrophils such as inhibition of cell adhesion, transmigration and cytokine secretion [106-109]. They have also been described to prevent ROS generation such as the formation of NO and $ONOO^-$ by neutrophils [109-115].

Moreover, LXA4 and ATL have been described to play a major role in the resolution of inflammation, together with the recently described resolvins and protectins, and these effects have been suggested to be mediated in part through binding to FPR2 [116, 117]. Resolution of inflammation is the process leading to homeostasis and restored tissue. Traditionally it was believed that resolution was a passive process, a result of withdrawal of proinflammatory events. Emerging evidence points out that resolution is a strictly regulated active process that already starts in the induction phase of the inflammation.

Lipoxins promote resolution by stimulating adherence and monocyte chemotaxis to site of inflammation without causing degranulation or release of reactive oxygen species [118]. It has been shown *in vitro* that lipoxin stimulates clearance of apoptotic neutrophils by monocyte-derived macrophages in a nonphlogistic manner [117, 119-122].

Consistent with a role for lipoxin promoting the resolution of inflammation are the observations that lipoxin-stimulated phagocytosis is associated with increased transforming growth factor-ß1 (TGF-ß1) and IL-10 release from macrophages, which further activates proresolving loops, as well as a decrease in the proinflammatory chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1/CCL2) release [120, 123]. Moreover, there are several examples demonstrating that impaired lipoxin synthesis is correlated with a more chronic inflammatory phenotype *in vivo* [124-126].

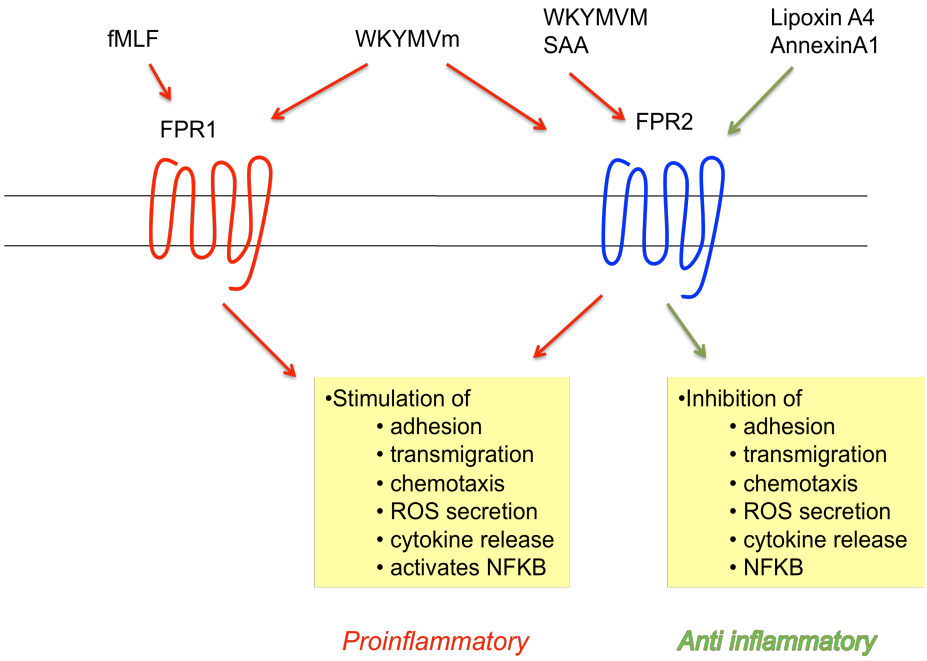


Figure 6. Activation of FPR1 leads to proinflammatory responses in neutrophils. Differential ligand-dependent signaling of FPR2 leads to either proinflammatory or anti-inflammatory responses.

3.3 Clinical implications of the FPR receptors and their ligands

FPR1 as well as FPR2 have been associated with several inflammatory and autoimmune disorders such as peridontitis (FPR1), allergy, Alzheimer's disease and RA (FPR2). Furthermore, ligands of FPR2, mediating proinflammatory as well as anti-inflammatory actions, are associated with inflammatory diseases such as RA (LXA4, annexin A1 and SAA), Alzeimer (annexin A1) and asthma, peridontitis, and colitis (LXA4) [43, 127-131].

Thus, receptor modulators of FPR1 and FPR2 as well as ligand mimetics may be of therapeutic value in a wide range of inflammatory diseases. One example is CGEN-855A, a synthetic FPR2 peptide agonist that was reported to exhibit efficacy in various acute *in vivo* models [132]. Moreover, several small molecule FPR2 agonists, such as the pyrazolone (compound 43) and FPR2 antagonists, quinazolinones (e.g. Quin-C7), have been developed by screening small molecule libraries. These compounds were reported to exhibit efficacy in established acute *in vivo* models such as inhibition of arachidonic-induced ear swelling in mice [133, 134].

Finally, one approach has been to develop lipoxin mimetics with improved metabolic stability. Several lipoxin analogues have been designed that have demonstrated comparable efficacy in vivo [135]. This approach has been used to develop 3-oxa-LXA4 analogues with potent anti-inflammatory profile and oral activity, that promoted the resolution of an established colitis model in mice [127].

3.4 Receptor-specific tools

As mentioned, the proinflammatory cellular responses downstream FPR1 and FPR2 exhibit large similarities between the two receptors. Investigations aimed to clarify differences in the downstream signaling from these receptors require tools in terms of receptor-specific agonists and antagonists. In the present study, fMLF, WKYMVM, cyclosporine H, WRWWWW and PBP10, previously described, were used as receptor-specific tools (Table 2).

Recently an inhibitor of FPR2 signaling, PBP10 was described. PBP10 is a gelsolin-derived peptide of 10 residues, conjugated to rhodamine B and has shown to be cell permeable in fibroblasts, platelets, melanoma cells and neutrophils. It is reported to be a strong PIP2 (phosphatidyl 4,5-biphosphate)-binding peptide and prevents chemotactic peptide stimulated actin assembly in platelets and neutrophils [136]. In detailed studies in neutrophils it was shown that PBP10 had a strong preference for inhibition of WKYMVM-stimulated over fMLF-stimulated signaling [137].

Ligand	FPR1	FPR2	Reference
Agonists			
fMLF	Kd=1 nM EC50=75 nM (ROS secretion)	Kd=1 μM	[95] [88]
WKYMVM	Unresponsive (calcium release)	EC50=2 nM (calcium release) EC50=75 nM (ROS secretion) Kd=160 nM	[88]
WKYMVm	1 nM (calcium release)	1 pM (calcium release)	[83]
Antagonists/Inhibitors			
cyclosporin H	IC50=120 nM (ROS secretion elicited by 20 nM fMLF)	IC50 > 10 μM (ROS secretion elicited by 20 nM WKYMVM)	[138]
WRWWWW	IC50>5 μM (ROS secretion elicited by 20 nM fMLF	IC50 > 200 nM (ROS secretion elicited by 20 nM WKYMVM)	[138]
PBP10	No inhibition of calcium response elicited by fMLF at 1-100 nM by stimulation with 1 μM PBP10	Calcium response elicited by 100 nM WKYMVM is reduced to 50 % by stimulation with 1 μM PBP10	[136, 137]

Table 2. Receptor-specific tools were used in present study to determine receptor-specific activation of FPR1 and FPR2 (paper I and II).

4 TARGETING CCL2 AND ITS RECEPTOR CCR2 IN SEARCH OF ANTI-INFLAMMATORY AND ANTI-FIBROTIC DRUGS

4.1 Basic biology of the CCL2/CCR2 axis

CCL2 (MCP-1-monocyte chemoattractant protein-1) was the first human C-C chemokine described and it belongs to a family with at least four members, CCL8 (MCP-2), CCL7 (MCP-3) and CCL13 (MCP-4). CCL2 is a potent chemoattractant for monocytes but also for T-lymphocytes and natural killer (NK) cells, recruiting these cells to sites of inflammation. CCL2 triggers the firm adhesion of monocytes to the vascular endothelium as well as migration through the vessel wall [65]. CCL2 may also be a key mediator of recruitment of monocytes from the bone marrow into the blood stream during homeostasis and with increased activity during inflammation [139]. The major source of CCL2 is hematopoietic cells, mainly monocytes and macrophages. CCL2 is also produced by endothelial and epithelial cells, fibroblasts, smooth muscle cells, astrocytes, mesangial cells and microglial cells.

Human CCL2 is a peptide of 76 amino acids some of which have been identified as critical for biological activity. The N-terminal residues have been shown to be important for receptor binding, induction of monocyte migration, and receptor desensitization [140-142]. It has been shown that the full-length CCL2 forms homodimers and some of the critical residues for dimerization were also localized in the N-terminus [140, 143]. CCL2 is secreted in two predominant forms of 9 and 13 kDa, respectively, differing in O-glycosylation sites. There is no difference in the biological activity between the two forms.
Expression of CCL2 is regulated at the transcriptional level by cytokines and growth factors such as TNFα, Interferonγ, PDGF as well as infectious agents and stress factors such as reactive oxygen species. Many of these responses are signaled via the proinflammatory factor NF-κB [142, 144].

CCL2 mediates its effects via CCR2, a GPCR with many similarities to those described above. There are two splice variants of the human CCR2 receptor, CCR2A and CCR2B that differ only in the C-terminus. CCR2A is the major isoform expressed on monocytes and vascular smooth muscle cells while CCR2B is expressed on monocytes and activated NK cells [65].

CCR2, the main receptor for CCL2, has dual roles as it mediates both pro and anti-inflammatory activities. The proinflammatory function is dependent on monocytes, APCs and T-cells, while the anti-inflammatory function is dependent on receptor expression in regulatory T-cells [65]. Moreover, there is emerging evidence indicating that the CCL2/CCR2 axis also is involved in T-cell immunity. CCL2 is associated with Th2 cell-mediated responses and has shown to induce the polarization of Th0 to Th2 cells in mice, possibly via upregulation of the Th2 cytokine IL-4 [65].

The murine CCR2 (JE receptor) shows 80% amino acid sequence identity with CCR2B [145]. In murine models CCL2 is corresponded with murine CCL2 (JE) that has a 55% identical amino acid sequence for the first 76 amino acids, but has also an extension of 49 amino acids in the C-terminus. Human CCL2 and JE have similar chemoattractant activity and thus they are structural and functional homologues [146]. Another agonist of the JE receptor, less potent, is CCL12 that shows 63 % sequence identity with human CCL2 [147].

4.2 Clinical implications of the CCL2/CCR2 axis

The CCL2/CCR2 axis is a major player in several pathologies associated with monocyte recruitment and it has been implicated in many diseases involving macrophages [65]. Mice genetically deficient in CCR2 as well as its ligand CCL2, exhibit reduced monocyte recruitment in disease models of peritonitis, tuberculosis and atherosclerosis [148]. Moreover, increased levels of CCL2 have been detected in synovial fluid from patients with RA, in bronchial epithelium of patients with idiopathic pulmonary fibrosis and in inflammatory skin diseases. CCL2 is also found in increased levels in macrophage-rich regions of atherosclerotic lesions and chronic acute hepatitis [149, 150].

The major role of the CCL2/CCR2 axis in inflammatory disease has many implications that make this receptor/ligand pair interesting as targets for new therapeutic drugs. Antagonizing the CCL2/CCR2 axis has been evaluated in several established animal models such as experimental autoimmune encephalomyelitis (EAE an *in vivo* model for MS), collagen-induced arthritis (CIA an animal mode of rheumatoid arthritis), bleomycin induced fibrosis, and in $CCL2^{-/-}$ and $CCR2^{-/-}$ knockout models. Results using all these models demonstrate both beneficial and non advantageous effects indicating a complex biology of blocking the CCL2/CCR2 axis using anti-CCL2 and anti-CCR2 monoclonal antibodies, modified peptide ligands and small molecule receptor antagonists. None of the evaluated agents have so far been approved, some of them have advanced as far as to a clinical phase II trial [150].

A humanized anti-CCR2 monoclonal antibody, MLN-1202, has been evaluated in clinical trials for MS, RA and atherosclerosis (phase II trials). The clinical outcome in the MS study was a small therapeutic effect (reduction of GD enhancing lesions) but unfortunately, there was no effect demonstrated in RA. A significant effect was observed in the clinical trial of atherosclerosis such as reduced CRP levels in patients with increased risk for atherosclerosis [150].

A small molecule CCR2 antagonist, MK0812, was evaluated in clinical trials with MS and RA patients (phase II) where no therapeutic effects were observed [150, 151]. Moreover, a study with a human monoclonal antibody, ABN912, targeting CCL2, was evaluated in a study with patients with active RA [152]. The clinical outcome was confusing. There was no therapeutic effect, rather the disease was aggravated in patients administered the highest dose. An explanation was suggested to be related to

the increased serum levels of CCL2 observed shortly after administration of the antibody. This was explained by immobilization of bound CCL2 and slow clearance of the CCL2-antibody complexes [150].

Development of modulators of the CCL2/CCR2 axis continue, but it is now focused on other therapeutic indications such as diabetes associated disorders [153, 154], neuropathic pain [155, 156], tumor diseases [142, 157, 158] and fibrosis [159-161] (paper III and IV).

4.3 7ND - a modified peptide antagonist

As mentioned the CCL2/CCR2 axis has been addressed with different approaches such as small molecule CCR2 inhibitors and antibodies against the ligand as well as the receptor. An additional strategy, discussed previously (page 19) is to use modified peptide ligands. The truncated variant of human CCL2 (1, 9-76), lacking amino acids 2-8, also named 7ND is an example of a peptide ligand agonist that is manipulated to obtain antagonistic properties. 7ND was first designed and characterized by Gong et al [162] and has been shown to have therapeutic effects in several animal in vivo models related to fibrosis and inflammatory diseases [163-167]. Interestingly, in all described examples, human 7ND was administered by gene transfection ensuring continuously transcribed protein. The reason may be that 7ND has a relatively short half life time in plasma and continuously expressed protein in the tissue will be compensating for degraded 7ND. Possible therapeutic effects obtained via administration of 7ND protein remain to be elucidated.

7ND is a well characterized inhibitor of the CCL2/CCR2 axis in human *in vitro* models, such as inhibition of calcium release and cell migration induced by CCL2 in human monocytes (THP-1 cell line). Furthermore, 7ND was shown to bind to the human CCR2 receptor expressed on THP-1 and CCR2-transfected cell lines with high affinty [141]. However, the main hypothesis put forward was that 7ND attenuates the function of CCL2 by heterodimerization and thereby inhibits CCL2 from binding to the receptor [143].

In contrast, 7ND is much less investigated in murine *in vitro* models and further analysis remain to clarify the mechanisms of the therapeutic effects *in vivo* (paper III).

4.4 CCL2/CCR2 in fibrosis

CCL2/CCR2 mediated activation of human fibroblasts

Various chemokines and their receptors have been associated with fibrosis and this is true also for CCL2 and its receptor. They have been reported to be involved in several fibrotic disorders such as systemic scleroderma (SSc) and idiopathic pulmonary fibrosis (IPF). SSc is a fibrotic disease of unknown aetiology affecting the skin and internal organs. Numerous groups have shown that increased levels of CCL2 are found in the skin of SSc patients. In early phases of the disease, the major source of CCL2 was reported to be infiltrating mononuclear cells. With progression activated fibroblasts become the major source of CCL2. In addition, increased levels of CCL2 have also been detected in the blood of SSc patients especially those in patients with pulmonary fibrosis [168].

One of the challenges in fibrosis research is the enormous complexity of the interactions and interplays between different cell types e.g. inflammatory leukocytes, various subtypes of fibroblasts and a large number of mediators such as cytokines/chemokines and their receptors. The CCL2/CCR2 axis is one component that may have implications in the initiation and the progress of fibrosis. One hypothesis is that CCL2 plays an important role as a chemoattractant of inflammatory cells in fibrotic tissue. Local expression of CCL2 in the skin of SSc patients may induce migration of inflammatory cells into the skin, mainly T-cells and macrophages, which in turn secrete profibrotic mediators leading to activation of resident fibroblasts and accumulation of extracellular matrix proteins and fibrosis. This is shown *in vitro* with fibroblasts explanted from SSc patients promoting recruitment of mononuclear leukocytes by release of CCL2. Migration was inhibited by CCL2 blocking antibodies showing that CCL2 was the major chemoattractant [168, 169].

CCL2 may also be a mediator of other cellular events involved in fibrosis, a hypothesis that has been intensively investigated, but still there are several questions that remain to be answered. Much of the research has focused on the role of CCL2 in fibroblast activation, the precise fibroblast subtype involved, and whether CCL2 mediates its effects via CCR2 or via not yet identified receptors. CCR2 is not found in all fibroblasts but has been detected in certain fibroblast subtypes. Fibrocytes, fibroblasts precursors, isolated from human and murine peripheral blood were shown to express leukocyte as well as mesenchymal markers on their surface and in addition, they expressed the CCR2 receptor [160]. CCL2 stimulation of human fibrocytes was found to increase fibrocyte proliferation as well as differentiation into fibroblast phenotype that may implicate their role in fibrotic disease.

Moreover, it has been shown that it is possible to isolate CCR2 positive fibroblast precursor cells (pericytes), explanted from skin tissue biopsies of early stage dcSSc (diffuse cutaneous systemic scleroderma) patients. In contrast, no CCR2 positive pericytes were isolated from late stage dcSSc or limited forms of SSc. CCR2 expressing fibroblasts were reported to over-express α-SMA and were able to

differentiate to myofibroblasts, but with differentiation they seemed to lose expression of CCR2. By specific inhibition of CCL2 or CCR2 using an anti CCL2 antibody or a receptor antagonist respectively, the base level of α-SMA was attenuated, suggesting an autocrine activation of the fibroblast via CCL2/CCR2 [170].

In contrast, from another study it was reported that dermal fibroblasts isolated from SSc patients were not activated by CCL2, no collagen I production was upregulated in fibroblasts from either healthy individuals or patients with SSc [169]. An interesting model to explain the role of CCL2 in fibrosis was proposed by the authors; increased levels of PDGF and IL-1β in fibrotic tissue induce an over-expression of CCL2 in dermal fibroblasts that in turn induces a Th2 differentiation of infiltrating Th0 cells. Th2 cells release large amounts of IL-4 that stimulates synthesis of collagen in fibroblasts [171].

As mentioned, CCL2/CCR2 has also been reported to have implications in fibrotic lung disease such as IPF and usual interstitial pneumonia (UIP). Fibroblasts explanted from biopsies of fibrotic and nonfibrotic patients were studied and it was shown that fibroblasts explanted from IPF/UIP patients exhibited upregulated levels of several fibrotic markers compared with those from nonfibrotic lung tissue. Moreover, fibrotic fibroblasts were hyperresponsive to stimulation with profibrotic mediators such as TGFβ and IL-13 but also CCL2, upregulating fibrotic markers multiple folds. In contrast, fibroblasts from non fibrotic patients were more or less unresponsive to these stimulus [172]. TGFβ1R, TGFβ2R, IL-13αR1 and IL-13αR2 were all upregulated in UIP/IPF fibroblasts which may explain the increased responsiveness of IL-13 and TGFβ. Interestingly no CCR2 transcript was found in any of the fibroblast cell lines but receptor protein was detected using immunohistochemical staining. Neutralization of CCL2 in unstimulated fibrotic fibroblasts had no effect on expression levels of fibrotic markers, however, CCL2 neutralization led to a moderate downregulation of TGFβ induced α-SMA and TGFβ1 gene expression indicating an interplay between TGFβ and CCL2. Whether this downregulation is mediated via CCR2 or via another mechanism was not shown.

CCL2/CCR2 in murine fibrotic in vivo models

The role of CCL2/CCR2 in murine fibrosis has also been studied in $CCR2^{-/-}$ and $CCL2^{/-}$ knockout mice. Pulmonary fibrosis is simulated in mice with intratracheal administration of FITC isomer I or bleomycin resulting in acute lung inflammation and extracellular matrix deposition [173, 174]. $CCR2^{-/-}$ knockout and wild-type animals were induced with bleomycin or FITC by intratracheal administration and it was shown that the knockouts were protected from lung fibrosis, exhibiting a significantly lower degree of inflammatory exudates and collagen in lung tissue. The protection from fibrosis was not primarily mediated by reduction of recruitment of inflammatory cells but rather by adjusting the balance of expression of cytokines such as TNFα [175]. In addition, fibroblast populations isolated from lung tissue of FITC-induced $CCR2^{-/-}$ and wild-type mice have been studied [175, 176]. A higher level of fibrocytes in wild-type mice was identified, indicating that CCR2 may be responsible for the recruitment of fibrocytes to FITC-induced lung tissue.

The $CCL2^{-/-}$knockout mouse was compared with the wild-type mouse in a skin fibrosis model. Mice were subcutaneously injected with bleomycin into the dorsal skin daily for four weeks. The $CCL2^{-/-}$ knockout was also in this case shown to be protected from fibrosis compared with the wild-type. While the wild-type developed an accumulation of collagen in the dermis and replacement of fat with connective tissue, the knockout exhibited less fibrotic tissue and preserved partially the subcutaneous fat. In this study it was also found that the $CCL2^{-/-}$ mouse exhibited a lower degree of infiltration of inflammatory cells such as neutrophils and monocytes into the inflamed tissue. Increased levels of CCL2 and TGFβ explain the increased recruitment of monocytes, activation of macrophages and differentiation of fibroblasts to myofibroblasts in wild-type mice. The authors also observed a shift in the balance between expression of MMPs and their inhibitors (TIMPs). As this balance is crucial for the turnover of the extracellular matrix, the shift in the balance observed in the $CCL2^{-/-}$ mouse might (at least partly) explain the protection against fibrosis [177].

5 AIM OF THESIS

The aim of the studies was to identify and characterize modulators of two chemoattractant receptors, FPR2 and CCR2 in order to investigate them as potential drug targets.

Paper I

The FPR2 receptor was previously described to mediate unique anti-inflammatory and proresolution properties through activation by receptor agonists of different chemical structures such as LXA4, annexin A1 and small molecules. The main goal of the study was to identify FPR2 agonists with lipoxin-like anti-inflammatory and proresolution properties. Another goal was to characterize selected hits in terms of selectivity and receptor hierarchy (FPR2 versus FPR1).

Paper II

The aim of this study was to screen for FPR2 antagonists and investigate if selected hits could be attributed to anti-inflammatory properties.
Another goal was to characterize the inhibitors in terms of anti-inflammatory properties and selectivity (FPR1 versus FPR2).

Paper III

The truncated form of CCL2 (1,9-76), also named 7ND, was described as an antagonist of human CCR2 in *in vitro* models. 7ND, administered by gene transfection was described to exhibit therapeutic effects in numerous *in vivo* models such as bleomycin-induced fibrosis. However, the mechanism of 7ND´s therapeutic effects in vivo has not been characterized and the interaction between 7ND and the murine CCR2 has not been described. One goal was to investigate if 7ND also inhibits the murine CCR2 receptor and the mechanism of such inhibition.
Another goal was to investigate if 7ND mediates antifibrotic effects directly on fibroblasts isolated from bleomycin-induced mice.

Paper IV

CCL2 has in several studies been described as a profibrotic mediator. It has been shown to upregulate synthesis of ECM proteins in fibroblasts, explanted from tissues of patients with fibroproliferative diseases. The aim of this investigation was to find out how CCL2 activates human fibroblasts of different origins in terms of fibrotic markers.

6 RESULTS AND DISCUSSION

6.1 Identification and characterization of FPR agonists- paper I

Screening for FPR2 agonists

The goal of the present investigation was to develop selective FPR2 agonists with lipoxin-like, anti-inflammatory and proresolution properties. As the potential ligands of FPR2 may be small molecules, eicosanoid lipids as well as peptides, different approaches are possible to apply in order to address this receptor. The selected strategy was to screen for FPR2 small molecule agonists that replicate the proresolution/anti-inflammatory properties reported for LXA4. The decision was primarily based on the high probability of success finding small molecule modulators to a GPCR Family A receptor and the extensive prior experience from our lab in working with this target family. Other considerations were the potential of the new agent to be orally available in addition to the relatively beneficial future production costs.
In this section preliminary and published data are discussed.

A small molecule library of 50 000 compounds were screened for FPR2 selective agonists. FPR2 transfected CHO cells (Chinese hamster ovary cells) were stimulated with each compound at a single concentration and the elicited intracellular calcium release was measured in FLIPR. 500 compounds, confirmed as agonist hits were counter screened in the parental cell line to exclude hits with non-specific receptor activity.

The five most potent receptor specific screen hits (compounds 5, 6, 8, 9 and 10), together with another five previously described FPR2 agonists, (compounds 1, 2, 3, 4 and 7) representing different chemical scaffolds, were selected for further analysis. ATL (aspirin-triggered lipoxin) and WKYMVm were included as reference compounds, representing differential signaling by the FPR2 receptor [97].
Structures of the compounds are described in paper I. Compound 1, 2 (enantiomers of Acadia C7) and 7, identified by screening small molecule libraries for FPR2 specific receptor activation in FPR2-transfected NIH3T3 cells, have been shown to dose dependently prevent hyperalgesia induced by carrageenan in a rat model (reference see paper I). Compound 3, a substituted quinazinolione (QuinC1), a FPR2 specific agonist has been described to be chemotactic and induce degranulation in neutrophils, but unable to stimulate superoxide production [134, 178]. A pyrazolone, compound 4, was identified by screening a small compound library for calcium release elicited by compound stimulated FPR2 and Gα15 co-transfected CHO cells. Compound 4 was reported to act anti-inflammatory; inhibiting fMLF and IL-8 induced cell migration in neutrophils. In addition, compound 4, named compound 43 in the reference, was also shown to be active *in vivo* and showed a dose dependent inhibition of ear swelling in mice following oral administration [133].

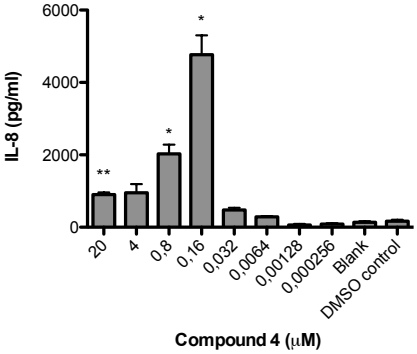
All twelve compounds were analyzed for dose dependent calcium release in FPR2/Gα16 transfected cells and EC50 values were determined. All compounds, except ATL were shown to induce calcium release in a dose dependent manner (Table 3) with EC50 values spanning from 6 nM to 230 nM. This interesting result of ATL was not that surprising; another group had previously made similar findings, that ATL is a very low affinity inducer of calcium release in FPR2-transfected cells [179].
Next step was to analyze receptor selectivity, FPR2 versus FPR1. It was determined for compound 1-10 in a calcium release assay in FPR1/Gα16 transfected cells. All compounds, except compound 9 induced calcium release with EC50 values from 9 nM to 9 μM The three most potent FPR2 agonists, compounds 4, 5 and 10 displayed different profiles, 4 and 5 were shown to be equipotent agonists of FPR1 and FPR2 while compound 10 was selective for FPR2 (Table 3).

Compound No	FPR2 Calcium EC50 (M)	FPR1 Calcium* EC50 (M)
1	3,4E-08	6,4E-06
2	3,2E-08	5,4E-06
3	3,9E-08	8,9E-06
4	4,2E-09	9,1E-09
5	6,0E-09	2,1E-08
6	3,9E-08	1,9E-07
7	4,5E-08	9,3E-06
8	1,0E-07	2,9E-06
9	2,3E-07	no response
10	6,0E-09	6,8E-06
ATL (Lipoxin)	> 5E-06*	not analyzed
WKYMVm	1,5E-09*	not analyzed

Table 3. Compounds 1-10 induce intracellular calcium release in FPR2 and FPR1 transfected CHO cells. CHO-K1 cells stably expressing the human FPR1 or FPR2 together with Gα16 were loaded with 3.6 μM Fluo4 AM (Molecular Probes, Eugene, OR), including 2,5 mM probenecid (SIGMA) for 60 min, at 37 C. In FLIPR 2 (Molecular Devices) cells were stimulated with compounds in a dose dependent manner from 20 μM to 3.4 pikomolar in 11 step and changes of cytosolic calcium were measured.
ATL (Calbiochem) (stock 709 μM in ETOH) was diluted in HBSS in 11 steps and cells were stimulated with ATL from 5 μM to 0.85 pM final concentrations. WKYMVm (Tocris) was diluted in HBSS and cells were stimulated with 100 nM to 1.7 pM final concentration. n=3, * Preliminary data

Analysis of pro- or anti-inflammatory properties

Next step was to analyze the agonists with respect to pro and anti-inflammatory properties. SAA, a proinflammatory mediator described as a FPR2 agonist was reported to induce IL-8 secretion in primary neutrophils, and stable lipoxin analogues were shown inhibit this response [92, 93]. Thus, compound-stimulated IL-8 release was considered to be an appropriate method to use for evaluation of our hits.
Compound 4-10, ATL and WKYMVm were analyzed for secretion of IL-8 in primary neutrophils. Compounds 4-7, 10 and WKYMVm were all shown to significantly induce IL-8 secretion (Figure 7 A). Accordingly, the most potent inducers of calcium release in FPR2 transfected cells, compounds 4, 5, 10 and WKYMVm were also the most potent inducers of IL-8 secretion. As expected, ATL at1 μM did not induce IL-8 secretion (Figure 7B) [97].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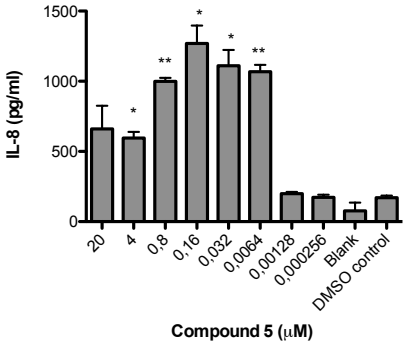
DMSO control
Compound 4 (μM)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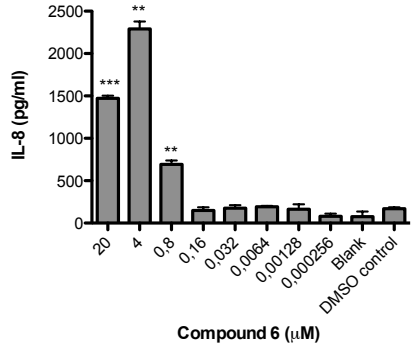
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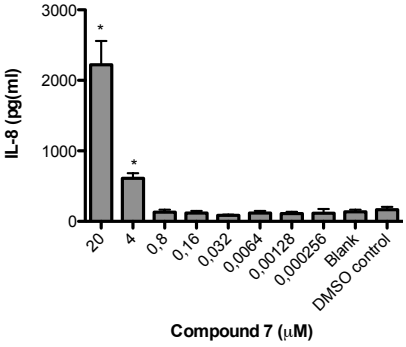
DMSO control
Compound 6 (μM)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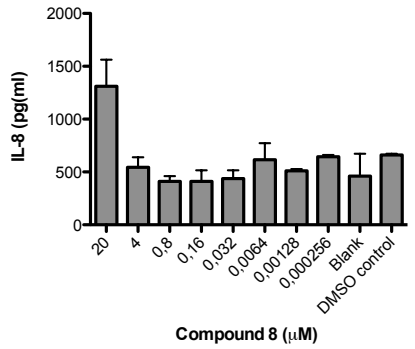
DMSO control
Compound 7 (μM)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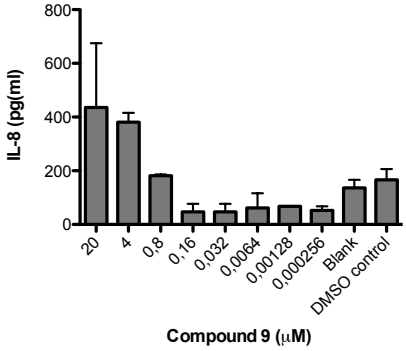
DMSO control
Compound 8 (μM)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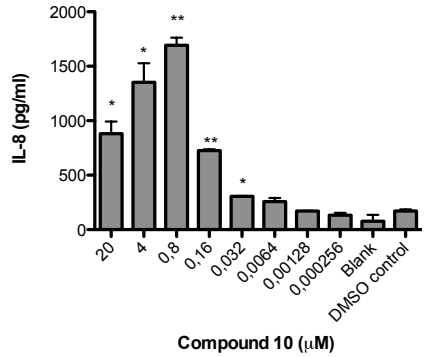
DMSO control
Compound 9 (μM)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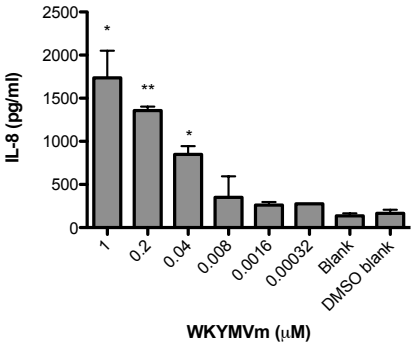
DMSO control
Compound 10 (μM)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2000
1500
1000
500
0
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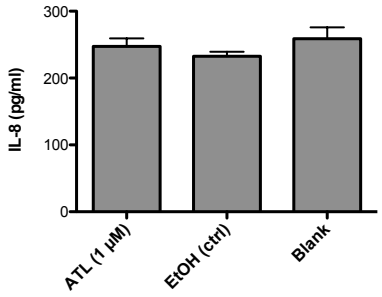
0,2
0,04
0,008
0,0016
0,00032
Blank
DMSO blank
WKYMVm (μM)

A.



B

Figure 7. Preliminary data. Compounds 4-7 and 10 induce Il-8 secretion in primary neutrophils (A). ATL at 1 μM did not induce IL-8 secretion compared to control (B).
Neutrophil granulocytes, prepared from buffy coat and isolated in a Ficoll-Paque gradient, were provided from 3H Biomedical (Uppsala, Sweden). Neutrophils were diluted to 5 x 10^6 cells/ml in assay buffer, 1xHBSS (GIBCO, 14180046) including HEPES (GIBCO, 15630049), and plated at a cell concentration of 400 000 cells/well in 96 well plates (Costar, 3599). Compounds dissolved in DMSO at a concentration of 10 mM were diluted in assay buffer to 200 μM and further stepwise to 2.56 nM. ATL (Calbiochem) (stock 709 μM in ETOH) was diluted in HBSS to 1 μM. Cells were stimulated with compounds at final concentrations from 20 μM to 0.256 nM and ATL at 1 μM for 4 hours. ETOH ctrl was 0.14 % ETOH in HBSS. DMSO blank was 0.2 % DMSO. After stimulation cells were centrifuged at 250 g for 5 minutes and IL-8 concentration in the supernatant was determined using a ready-to-use HTRF kit (CisBio, 62IL8PEB). The FRET read-out, proportional to the IL-8 concentration, was measured in an EnVision 2102 multilabel reader (Perkin Elmer). IL-8 concentration was plotted as a function of compound concentration. Values are the mean± SEM.*,P<0.05,**,P<0.01,***,P<0.001.

To further confirm the proinflammatory character of the compounds, they were analyzed for induction of NADPH oxidase activity (paper I). Isolated human neutrophils were stimulated with compound (1-10) and secretion of superoxide anions was measured using a previously described method (isoluminol-enhanced chemiluminescence) [180]. fMLF, the reference compound of this study, together with compounds 1-10, were all shown to induce NADPH-oxidase activity in primary neutrophils. The same correlation that was observed between calcium release in FPR2 transfected cells and IL-8 release in neutrophils, was observed in the NADPH oxidase analysis. Compounds 8 and 9 induced a respiratory burst only in TNFα-primed cells and at concentrations higher that 1 μM, whereas the compounds 1, 2, 3, 6 and 7 triggered also resting cells to produce superoxide anions (data not shown). The three most potent compounds 4, 5 and 10 induced a robust NADPH oxidase response in resting cells (Figure 8). ATL was not included in this study since it had previously been reported to not induce ROS secretion in human neutrophils [97, 181].

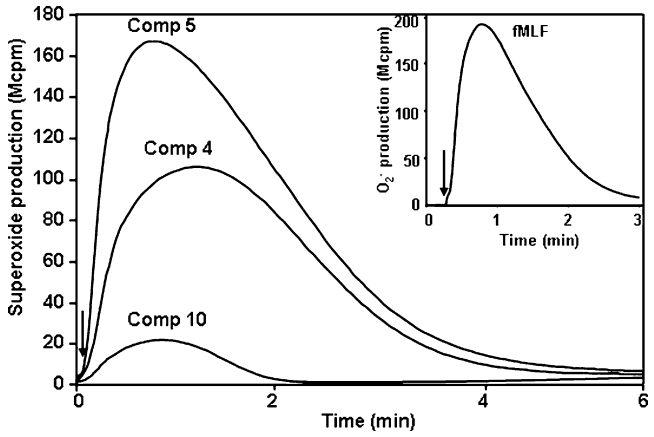


Figure 8. Activation of NADPH oxidase in neutrophils. Cells were preincubated for 5 minutes at 37^0 C and then challenged with 1 μM of compound 4, 5 and 10 or 100 nM fMLF.

Furthermore, compounds 4, 5 and 10 were analyzed for two other proinflammatory events in neutrophils, mobilization of surface CR3 and induction of cell migration in the same cells. Accordingly, compound 5 was the most potent inducer of chemotaxis while compound 4 was the most potent inducer of CR3 mobilization.

Taken together, our results demonstrated that compounds 4-7 and 10 were all able to induce proinflammatory actions in primary neutrophils. In addition, there was a correlation between the potency of the compounds in the calcium release assay in FPR2 transfected cells and their ability to induce proinflammatory events in neutrohils. To our surprise, compound 4, described as an anti-inflammatory compound in both *in vitro* and *in vivo* models, demonstrated another profile in our hands. Compound 4 was, in contrast to described data [133], an equipotent inducer of calcium release in FPR2 and FPR1 transfected cell lines and in addition, able to induce proinflammatory activities in neutrophils.
Based on these findings, the goal of the project was changed. The new goal was to further characterize compounds 4, 5 and 10 in order to understand via which receptor, FPR1 or FPR2, that these compounds mediate their effects in neutrophils.

Receptor selectivity-FPR1 versus FPR2

Receptor selectivity in neutrophils was determined by measurements of compound-induced NADPH oxidase activity in presence of receptor specific inhibitors/antagonists, PBP10 and WRWWWW (FPR2) and cyclosporine H (FPR1) (described in 3.4). Data showed that NADPH oxidase activity induced by compounds 4, 5 and 10 was completely blocked by cyclosporine H demonstrating that all compounds were activating FPR1. This is in line with calcium release data in transfected cell lines except for compound 10. Furthermore, the FPR2 specific inhibitor WRWWWW was shown to be a partial inhibitor of compound induced responses, but surprisingly, PBP10 did not block the responses at all. These data led to further investigations.

To elucidate the preferred receptor of compounds 4, 5 and 10 in primary neutrophils, homologue desensitization studies were performed (paper I). Neutrophils were first challenged with fMLF followed by stimulation with compounds and measurement of NADPH oxidase activity. For all three compounds the responses were completely desensitized. When fMLF and the compounds were added in reverse order the responses were still completely abolished. These data demonstrate that compounds 4, 5, and 10 were all activating FPR1 at this concentration. When neutrophils were stimulated with WKYMVM followed by compound stimulation, the responses were completely abolished. In contrast, when WKYMVM and compounds were added in reverse order, the responses were partially inhibited. These results indicate that compounds 4, 5 and 10 activate both FPR1 and FPR2 but may have a preference for FPR1.

Taken together, this study showed that compounds 4 and 5 were high potent agonists of FPR1 as well as FPR2 in a calcium release assay in transfected cell lines. In contrast, compound 10 demonstrated a much higher affinity to FPR2 than FPR1 in transfected cells. However, in primary neutrophils where both receptors were present, the receptor preference was slightly different. Use of receptor specific antagonists and desensitization studies indicate that all three compounds activate both receptors for induction of NADPH oxidase activity, but may use FPR1 as the preferred one.

6.2 Identification and characterization of selective FPR inhibitors - paper II

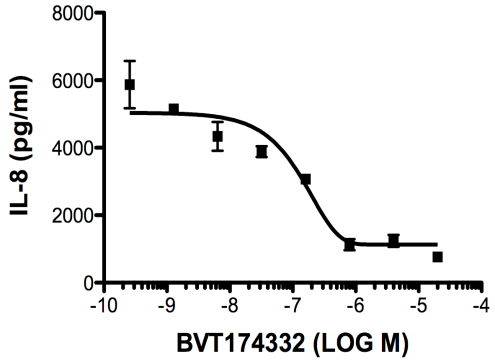
Screening for FPR2 antagonists

The first goal of this study was to evaluate if screening a small molecule library for FPR2 antagonists would be a feasible approach for identification of new anti-inflammatory compounds. A successful example is the small molecule FPR2 antagonist that was recently described to exhibit anti-inflammatory effects *in vivo* [134, 182]. That data, together with the outcome of the FPR2 agonist screen supported this approach, screening a small molecule library (20 000 compounds) for FPR2 antagonists. The second goal was to characterize screen hits in order to elucidate receptor selectivity in neutrophils.

FPR2/Gα16 transfected CHO cells were stimulated with a small molecule library and challenged with 2 nM WKYMVm followed by measuring the calcium release in FLIPR (method described in paper I). Few screen hits were identified. However, two structurally related compounds (salicyl-anilides), BVT015825 and BVT174332, shown to inhibit WKYMVm-induced calcium release in a dose- dependent manner, were selected (data not shown).

Analysis for anti-inflammatory properties

Selected hits were further analyzed for anti-inflammatory properties. BVT015825 and BVT174332 were analyzed for inhibition of LPS-stimulated and WKYMVm stimulated IL-8 secretion in neutrophils. BVT015825 and BVT174332 were shown to inhibit WKYMVm -stimulated as well as LPS-stimulated IL-8 secretion in human neutrophils. Interestingly, in opposite to what had been described previously, ATL did not exhibit any inhibitory effects on LPS-stimulated IL-8 secretion (preliminary data, figure 9) [183].



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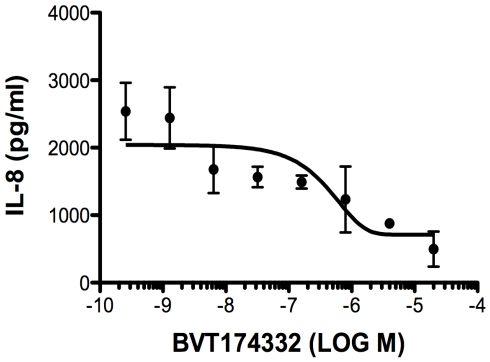

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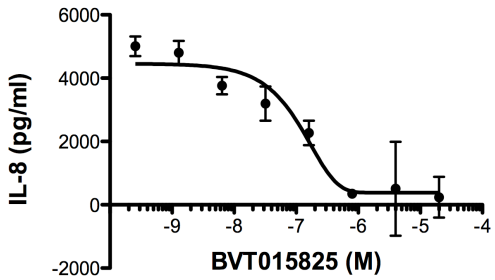

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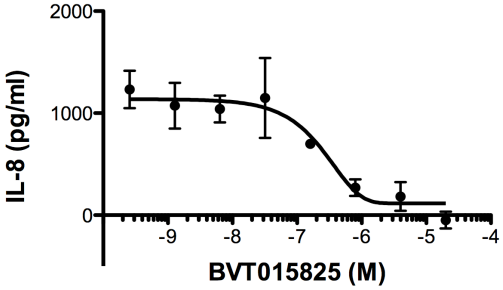

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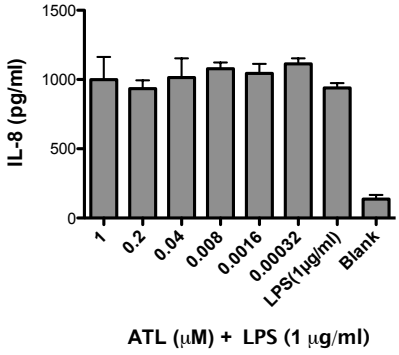


E

Figure 9. Preliminary data. Compounds selected from a small library screen, BVT174332, BVT015825 together with ATL were evaluated for inhibition of LPS (A, C and E) and WKYMVm (B, D)-stimulated IL-8 secretion. Preparations of neutrophils as well as measurement of IL-8 were performed according to the method described in Figure 7. Cells were stimulated at final concentrations from 20 μM to 0.256 nM for 15 minutes with compounds prior to stimulation with 1 μg/ml of LPS (Sigma, L4516) or 1 μM of WKYMVm (Innovagen, SP-WPEP-1) or controls for another 4 hours. IL-8 concentration was plotted as a function of compound concentration (n=2).

Receptor selectivity-FPR1 versus FPR2

To further explore the structure activity relationship and hopefully be able to identify new selective inhibitors, a set of 44 structurally similar compounds to BVT174332 and BVT015825 was selected. These compounds were analyzed for inhibition of NADPH oxidase activity in primary neutrophils, in presence of fMLF or WKYMVM in order to determine potency as well as receptor selectivity (FPR2 versus FPR1) (paper II). One of these, BVT173187, was shown to be the most selective compound. It was selective for FPR1 signaling, inhibiting fMLF stimulated release of reactive oxygen species with an IC50 of 0.1 μM. When BVT173187 was compared with cyclosporine H it was shown that the IC50 values of the two compounds were the same. Selectivity was determined at 1 μM of BVT173187 in presence of either 100 nM of fMLF or 100 nM of WKYMVM. Inhibition induced was defined as the ratio of the peak values of the agonist responses in presence or absence of antagonist according to a recently described tool for analysis of receptor specific events [184].
The ratio fMLF/fMLF+BVT173187 was 20,7 while the ratio for WKYMVM/WKYMVM+BVT173187 was 1,3, suggesting a strong preference for inhibition of FPR1 signaling (Figure 11). However, BVT173187 was also inhibiting NADPH oxidase triggered by C5a and IL-8 indicating that it was acting also via the CXCR1/ CXCR2 and C5a receptors. BVT015825 did not inhibit superoxide release induced by fMLF or WKYMVM. BVT174332 was a partial inhibitor of superoxide release induced by fMLF (Table 4).

Taken together, the structurally related compounds BVT174332, BVT015825 and BVT173187, all inhibited proinflammatory actions in neutrophils. Interestingly, they are structurally related to IMD0354 (Figure 10) a salicyl-anilide that was described as a cell permeable IKKβ (inhibitor of nuclear factor-κB kinase subunit) inhibitor and has demonstrated anti-allergic and anti-inflammatory effects in a mouse model of allergic inflammation [185]. This indicates that also BVT173187, BVT174332 and BVT015825 may be taken up by the cell acting downstream the receptors. Thus, it remains to determine in further studies the exact mechanisms of action of these compounds.

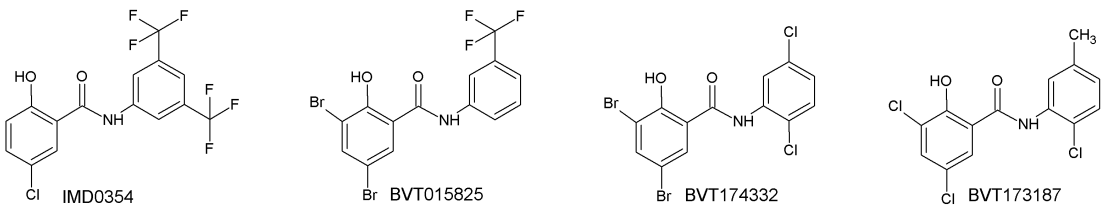


Figure 10. Salicyl-anilides exhibit anti-inflammatory actions in vivo (IMD0354) and in vitro (BVT015825, BVT174332 and BVT173187).

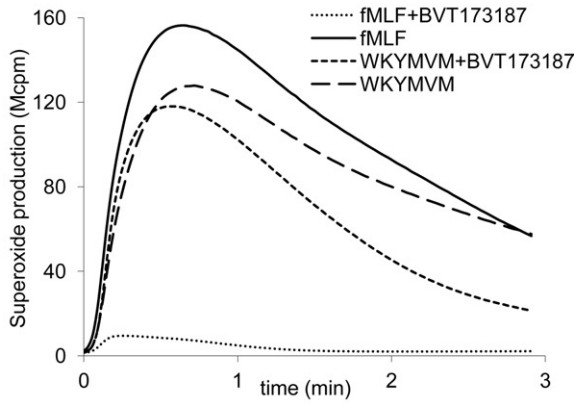


Figure 11. FPR1 is the preferred receptor over FPR2. Neutrophils were incubated in presence or absence of 1 μM BVT173187 and challenged with 100 nM fMLF or 100 nM WKYMVM and the production of superoxide anion was monitored. n=4

	Ratio
BVT174332	
fMLF/WKYMVM	1.1
fMLF/fMLF+BVT174332	2.7
WKYMVM/WKYMVM+ BVT174332	0.9
BVT173187	
fMLF/WKYMVM	1.2
fMLF/fMLF+BVT173187	20.7
WKYMVM/WKYMVM+ BVT173187	1.3
BVT015825	
fMLF/WKYMVM	0.9
fMLF/fMLF+BVT015825	1.2
WKYMVM/WKYMVM+ BVT015825	0.7

Table 4. BVT174332 and BVT173187 were shown to inhibit fMLF-stimulated superoxide in neutrophils. Inhibition and selectivity was determined for BVT0158251, BVT17433 and BVT173187 in presence of either fMLF or WKYMVM. Inhibition induced was defined as the ratio of the peak values of the agonist responses in presence or absence of antagonist according to a recently described tool for analysis of receptor specific events [184].

6.3 7ND-a modulator of the human but not the murine CCR2-paper III

7ND, CCL2 (1, 9-76) has been administered with gene transfection and shown to have therapeutic effects in several animal *in vivo* models related to fibrosis and inflammatory diseases [163-167]. Thus, development of a peptide ligand, based on 7ND was considered to be an attractive approach targeting the CCL2/CCR2 axis.
The goal of this study was to characterize how 7ND interacts with CCL2 and CCR2 of murine origin.

7ND has previously been shown to bind human CCR2 with high affinity and in addition, antagonize CCL2 stimulated chemotaxis and calcium release in human monocytes [141]. However, the mechanism with which 7ND inhibits CCL2 stimulated chemotaxis of human monocytes is not fully understood. One hypothesis is that 7ND attenuates the CCL2 mediated effects by heterodimerization with soluble CCL2 and thereby blocks the binding of CCL2 to the receptor. Formation of CCL2/7ND heterodimers was demonstrated by SDS-PAGE analysis of coprecipitated FLAG-labeled 7ND and radiolabeled human CCL2 in presence of an anti-FLAG antibody. In line with this, additional data indicate that 7ND was able to inhibit CCL2 in solution, but not cross-linked CCL2, from inducing cell migration of the THP-1 cell line [65, 140, 143].

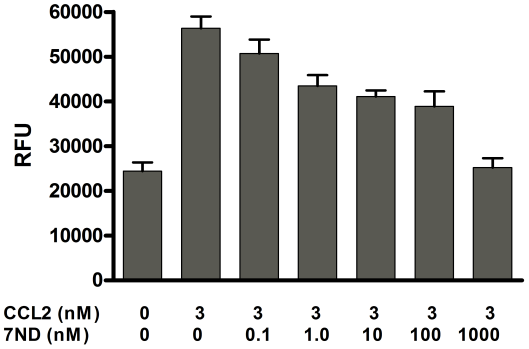


Figure 12. Inhibition of CCL2- induced cell migration by 7ND was determined using Transwell 96-well microplates of 5 mm pore size. Human CCL2 (3 nM) (EC70 challenge dose) or buffer was added to the lower chambers. 7ND at concentrations between 0.1 and 1000 nM or buffer, resuspended with THP-cells (106 cells/ml) were added to the upper chambers and allowed to migrate for 4 h in humidified atmosphere (5% CO2) at 37 8C. Relative fluorescence (RFU), as a measure of migrated cells, was plotted as a function of 7ND concentration (n = 3) (paper III*)*

However when it comes to the mechanisms with which 7ND mediates it effects in described *in vivo* models, much less is investigated. Although it is generally assumed, there are no data showing that 7ND is acting by a mechanism involving CCL2/CCR2 in these models. The hypothesis of 7ND forming heterodimers with murine CCL2 was

described but has not been verified [163]. Thus, it still remains to show if 7ND mediates its effects via inhibition of murine CCL2 stimulated effects in murine models.

Firstly, we have confirmed that 7ND inhibits human CCL2 stimulated calcium release as well as cell migration of a human monocyte cell line, THP-1 (Figure 12) (paper III).
Next step was to investigate if 7ND inhibits mCCL2-stimulated effects in cells expressing murine CCR2. As we were not able to identify a murine cell line with high enough levels of endogenously expressed CCR2, we used a mCCR2 transfected cell line for these studies (paper III). 7ND was at first evaluated in a functional assay; inhibition of mCCL2 stimulated calcium release. Surprisingly, it was shown that 7ND inhibits mCCL2 stimulated calcium release with very low affinity (data not shown).

Next step was to investigate if 7ND binds to mCCR2. In a displacement assay using ^{125}I-JE it was confirmed that mCCL2 binds to mCCR2 with high affinity [145]. In contrast, 7ND was confirmed, in accordance with calcium release data, to be a very low affinity binder of murine CCR2 (Figure 13) (paper III).

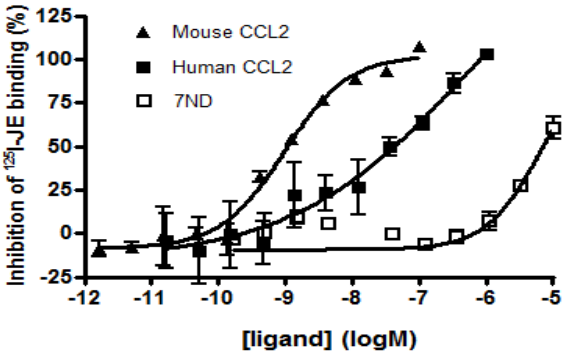


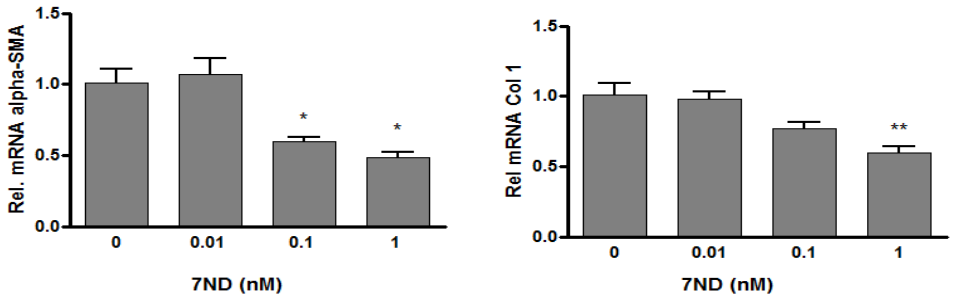
Figure 13. Displacement of ^{125}I-JE binding to mouse CCR2. The three peptides mCCL2, hCCL2 and 7ND displaced ^{125}I-mCCL2 with pKi values of 9.33 ± 0.22 (0.46 nM, n=4), 7.22 (60 nM, n=1), and >6 (>1 uM, n=4), respectively.

As mentioned, the hypothesis put forward is that 7ND inhibits CCL2 mediated effects by heterodimerization of the two ligands [143]. If this hypothesis could be confirmed, it would explain how 7ND interacts with mCCR2, and clarify the mechanisms with which 7ND mediates its effects in *in vivo* models.
In order to study the interaction between 7ND and mCCL2, mCCL2 was immobilized on a dextran-free chip and binding kinetics was studied by the surface plasmon resonance (SPR) technology [186]. Heterodimerization was analyzed over a wide range of concentrations of 7ND and showed that 7ND binds to murine CCL2. However, the interaction between the two ligands was weak; the dissociation constant of the 7ND/mCCL2 complex was determined to 670 nM (paper III). To be able to confirm these data and determine the stoichiometry, further experiments remain, such as studies of the CCL2/7ND complex in solution.

Taken together, 7ND dimerizes with murine CCL2 with low affinity and moreover, it binds with low affinity to the murine CCR2. Accordingly, we found that 7ND inhibits CCL2 stimulated calcium release in mouse CCR2 transfected cell line with low affinity

as well. From these data we concluded that 7ND mediates its effect via CCR2 in mice with low affinity and that 7ND probably acts via an alternate pathway with high potency in described murine models.

Based on these data it was concluded that 7ND might have another role than inhibition of CCR2 expressing leukocytes *in vivo*. Based on the fact that CCL2 may be involved in activation of fibroblasts in fibrotic tissue [170, 172] and that the effects of 7ND on isolated fibroblasts are not well described, the effects of 7ND on murine fibroblasts isolated from bleomycin-induced mice were evaluated. Fibroblasts were isolated from lung tissue of bleomycin-induced fibrotic mice [173] and stimulated with subnanomolar concentrations of 7ND. The concentration range of 7ND was based on the plasma concentrations measured in a bleomycin-induced mouse model that had been 7ND gene transfected [163]. Interestingly, it was found that 7ND significantly downregulated markers associated with activated fibroblasts such as α-SMA, collagen I (Figure 14), collagen III and fibronectin. No CCR2 mRNA was detected in isolated fibroblasts indicating that these effects were independent of the receptor (paper III).



A B

Figure 14. 7ND inhibits mRNA expression levels of fibrotic markers in murine lung fibroblasts in a concentration dependent manner. Levels of mRNA transcripts were detected by real-time PCR. (A) a-smooth muscle actin (B) Collagen I. Representative data of 1 of 3 experiments are presented and values are the mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.005.

6.4 CCL2 mediated downregulation of human fibroblasts - paper IV

As mentioned, previously reported results indicate that CCL2 promotes activation of fibroblasts explanted from fibrotic tissue of skin as well as lungs, while fibroblasts originating from non fibrotic tissue seem to be unresponsive [170, 172]. However, several questions remain to be answered and further research is needed to understand the role of CCL2 in activation of fibroblasts. Divergent results [169] in these *in vitro* studies may be explained with differences in experimental set-up such as preparation of the isolated cell lines, which may result in outgrowth of different fibroblast subtypes. Moreover, cell cultures in low passages may still contain mononuclear cells that secrete profibrotic mediators, e.g. TGFß, that stimulate fibroblasts.

Another key question is whether CCL2 activates fibroblasts via CCR2 or other pathways. CCR2 expression has been attributed to fibroblast precursor cells, fibrocytes and pericytes, and with increased cultivation passages *in vitro*, cells transform and

CCR2 expression may diminish [176]. Moreover, fibroblasts explanted from patients in different stages of the disease, e.g. SSc, could have different status, e.g. with respect to CCR2 expression, and respond differentially.

To further broaden our understanding of the effects of CCL2 on the fibroblast phenotype in general, three established fibroblast cell lines were studied (paper IV). The human fetal lung fibroblast (HFL-1) cell line is a commercially available cell line used for studies of the role of CCL2 in fibrosis. HFL-1 was described to respond to CCL2 stimulation with IL-6 secretion and increased survival following staurosporine-induced apoptosis. The hypothesis put forward was that CCL2 contributes to fibrosis by production of the prosurvival cytokine IL-6 and inhibiting apoptosis in fibroblasts [187]. Using the HFL-1 cell line some interesting findings were made. When they were stimulated with CCL2, correlating decreased levels of transcripts of fibrotic markers such as collagen I and α-SMA were observed (paper IV). This was unexpected and led us to explore another two fibroblast subtypes, primary preadipocytes and primary human pulmonary fibroblasts (HPF) isolated from non fibrotic patients. Stimulation of preadipocytes with CCL2 also resulted in decreased levels of collagen I and α-SMA (Figure 15). Interestingly, neither CCR2 transcript nor protein was detected in any of these three fibroblast cell lines, indicating that CCL2-mediated effects in HFL-1 and preadipocytes were independent of CCR2. Human pulmonary fibroblasts were unresponsive to CCL2 in regards to collagen expression in accordance with results reported by Murray et al [172]. These results confirmed that CCL2, at least in *in vitro* models, activates fibroblasts differentially depending on origin and status of the cell.

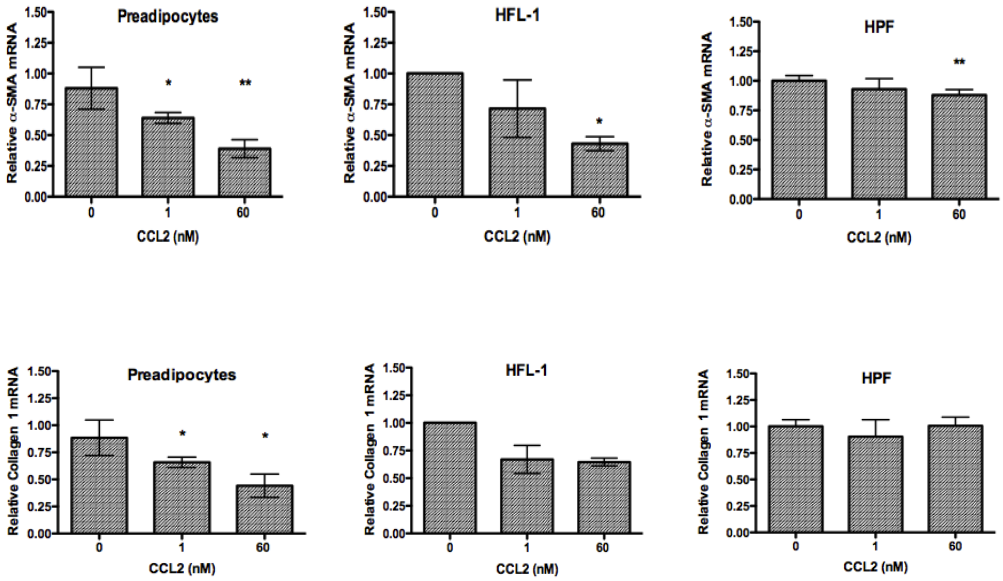


Figure 15. CCL2 inhibits mRNA expression levels of fibrotic markers in human fibroblasts in a concentration dependent manner. HPF, HFL-1 and preadipocytes were stimulated with human CCL2 (1 and 60 nM). Levels of mRNA transcripts were detected by real-time PCR. A) α-smooth muscle actin, B) Collagen 1.
Values are the mean±SEM.*,P<0.05,**,P<0.01,***,P<0.001.

It is clear that CCL2 plays a role in the regulation of collagen turnover and ECM remodelling. Opposite to the described profibrotic effects in fibroblasts explanted from fibrotic patients, CCL2 has also been associated with downregulation of procollagen I

and TGFβ II receptor (TGFβRII) in skin fibroblasts. Ultraviolet light (UVB) exposure to skin fibroblasts induces upregulation of CCL2 and MMP1 transcripts and induces downregulation of mRNA expression of procollagen I and TGFβRII. MMP1 is responsible for degradation of procollagen I, and decreased levels of TGFβRII contribute to reduced TGFβ-stimulated expression of ECM [188]. In line with these results, Mithell et al presented data supporting the antifibrotic role of CCL2. $CCR2^{-/-}$ knockout mice demonstrated an attenuated resolution of CCL4-induced liver fibrosis compared to the wild-type mouse and these observations were shown to be caused by reduced levels of MMPs (MMP2 and MMP13) and persisting levels of TIMPs [189]. These studies support the conclusion that CCL2 is associated also with antifibrotic responses.

The factors that regulate the way fibroblasts respond to proinflammatory (profibrotic) stimuli still remain to be explored. It was recently described that predipocytes and dermal fibroblasts, attributed to very similar fibroblast phenotypes, responded in an opposite manner to proinflammatory stimuli such as LPS and TNFα. While preadipocytes improve anti-inflammatory activities, dermal fibroblasts enhance inflammation [190].

Taken together, these studies confirm that resident fibroblasts from non fibrotic individuals do not express CCR2. In addition, data confirm that primary resident lung fibroblasts from healthy individuals are unresponsive to CCL2. Interestingly, two other fibroblasts responded with downregulation of fibrotic markers. In order to better understand the consistency of these observations, essential follow-up experiments include extended time studies with an increased range of CCL2 concentration. To understand the relevance of these data it is valuable to analyze the underlying mechanisms and relate them to those of CCL2 mediated actions in a profibrotic context. Neutralization of CCL2 with a blocking antibody would further confirm if these effects are directly mediated by CCL2.

6.5 Modulation of fibrotic markers in CCL2 and 7ND stimulated fibroblasts- paper III and IV

As mentioned, CCL2 is described in several examples as a profibrotic factor acting via CCR2, that stimulates recruitment of inflammatory cells and precursors of fibroblasts into fibrotic tissue in human as well as murine models. 7ND was shown to be an antagonist of human CCR2 inhibiting calcium release and recruitment of inflammatory cells in vitro and in vivo. In contrast, 7ND exhibited low affinity to murine CCR2 but was still demonstrating inhibitory effects in murine fibroblasts, downregulating fibrotic markers. Interestingly, in accordance with 7ND, CCL2 stimulated similar effects in two human fibroblast cell lines of different origin, HFL-1 and preadipocytes. Accordingly, none of the fibroblasts, either of murine or human origin, were expressing the CCR2 receptor (Figure 16). Apparently CCL2 and 7ND induce antifibrotic signaling in fibroblasts independently of CCR2 and thereby excluding uptake of CCL2 by non-specific scavenger receptors. In order to identify the alternate pathways of CCL2 and 7ND, further investigations are needed. Stimulation of fibroblasts in presence of

pertussis toxin would give some information whether another Gαi coupled chemokine receptor is involved or not. It has been shown previously that low concentrations of CCL2 induce cellular events in pancreatic beta cells as well as in vascular smooth muscle cells independently of CCR2 but via another pertussis toxin sensitive pathway [191, 192]. Panning of the chemokine receptor repertoire of the used fibroblasts would give us an idea about what other receptors that might come into play and from that information one may exclude one after the other. If this still does not give the answer, target fishing will be the next step [193].

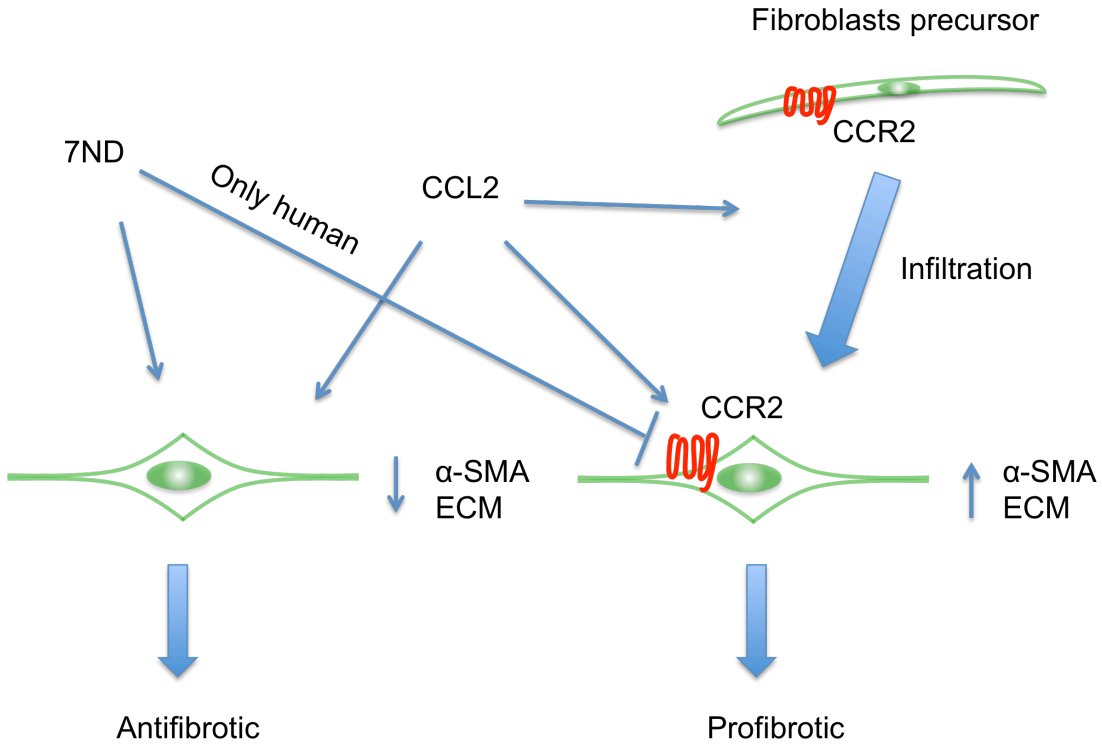


Figure 16. CCL2 mediates infiltration of CCR2 positive fibroblast precursors to fibrotic tissue. Emerging evidences indicate that CCL2 also promotes profibrotic activation of subtypes of fibroblasts via CCR2. In addition, new observations indicate that CCL2 and its truncated analogue 7ND (1,9-76), promote antifibrotic activities in fibroblasts of two different origins, HFL-1 and preadipocytes independently of CCR2 (paper III and IV).

7 CONCLUDING REMARKS

In present studies FPR2 and CCR2 have been investigated as potential targets in search of new anti-inflammatory and antifibrotic drugs. One goal was to identify anti-inflammatory compounds by screening a small molecule library for FPR2 agonists. Promising reference compounds described as efficient anti-inflammatory agents *in vitro* and *in vivo* such as a pyrazolone, compound 4, was evaluated. In our hands the pyrazolone exhibited, in similarity with our selected most potent agonist hits, proinflammatory properties in several *in vitro* models. Moreover, the pyrazolone was also previously described to be relatively selective for FPR2 but we could show that it induced calcium release equally well in FPR1 as in FPR2 transfected cells. In addition, studies of receptor selectivity and hierarchy in primary neutrophils rather indicated a possible preference for the FPR1 receptor. These conflicting data brings insight into the fact that chemoattractants and their receptors may act differentially depending on what model is used. ATL described as a potent inhibitor of neutrophil activation in several *in vitro* models was, in our neutrophil model inactive. In line with this result, other conflicting data about the effects of LXA4 in neutrophils have recently been published [98].

FPR2 transfected cells were screened for receptor antagonists as well and a small group of structurally related compounds were identified that were shown to inhibit LPS-stimulated IL-8 secretion in neutrophils. By using receptor specific tools, it was possible to show that one of them, BVT173187, demonstrated a clear preference for inhibition of FPR1 signaling, attenuating secretion of reactive oxygen as well as mobilization of CR3 in neutrophils. The complete mechanism of action of the compound though, remains to be determined. Future work will show if this compound, besides its therapeutic potential, could be a valuable tool in disclosing differences in signaling downstream the FPR1 and FPR2 receptors.

CCL2 and its receptor CCR2 are major players for recruitment of monocytes to inflammatory sites. There are also emerging evidences for their involvement in development of fibrosis; CCL2/CCR2 axis is reported to promote fibrosis in several *in vitro* and *in vivo* models. Interestingly, observations have also been made that demonstrate antifibrotic actions by CCL2 in fibroblasts originating from non fibrotic patients. These effects were shown to be independent of CCR2.

To address CCR2 in order to develop antifibrotic agents the selected approach was to evaluate the truncated form of CCL2 (1,9-76), 7ND that is described as a CCR2 antagonist and has demonstrated efficacy in several *in vivo* models, e.g. bleomycin-induced lung fibrosis. Surprisingly, it was found that 7ND is a very low affinity binder of the murine CCR2 homologue leading to the hypothesis that 7ND mediates its therapeutic effects in murine *in vivo* models independently of CCR2. From studies of 7ND stimulated murine fibrotic lung fibroblasts, some interesting findings were made, that possibly can explain the pharmacological properties of 7ND in mice models. Our hypothesis is that 7ND acts as an inverse agonist that down-regulates the basal levels of

α-SMA and extracellular matrix proteins, suggesting that 7ND may protect from development of fibrosis independently of CCR2.
Notably, similar anti-fibrotic properties were observed for CCL2 on human CCR2 negative fibroblasts. Although we are only at the beginning of understanding such a CCR2 independent pathway, a reasonable hypothesis could be that 7ND and CCL2 share the same anti-fibrotic mechanism in this context, whereas CCL2 is a mediator of profibrotic responses via CCR2.

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