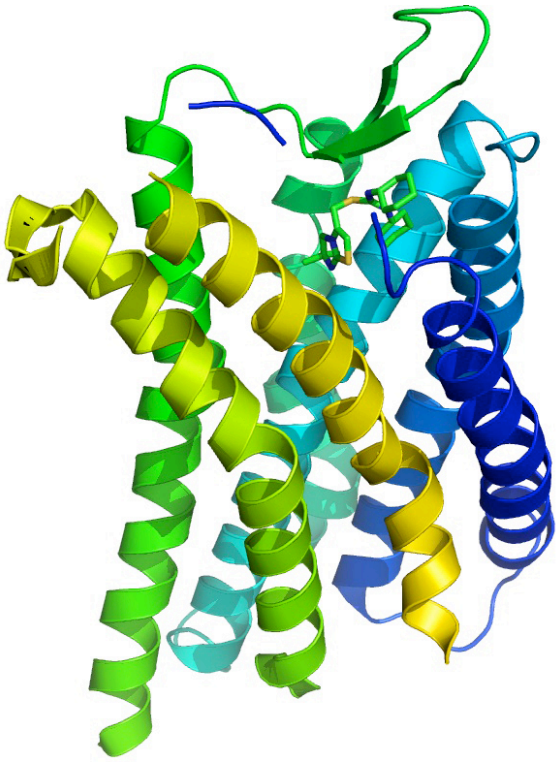
Elucidation of the binding site of antagonists for the human chemokine receptor CCR2b



(Crystal structure of CXCR4) [1]

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Con immenso affetto a voi

cari mamma e papà

“ Above all, don’t fear difficult moments. The best comes from them ”.

– Rita Levi Montalcini

(1909- 2012) Italian-American scientist, 1986 Nobel Prize winner in Physiology or Medicine

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List of abbreviation

7TM	Seven transmembrane receptor
BCA	Bicinchoninic Acid
CCL2	Chemokine Ligand 2
CCR2	Chemokine Receptor 2
CHAPS	3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonate
DMEM	Dulbecco Modified Empty Medium
DRG	Dorsal Root Ganglia
DTH	Delayed Type Hypersensitivity
EAE	Experimental Allergic Encephalomyelitis
GAGs	Glycosaminoglycans
GDP	Guanosine Diphosphate
GPCRs	G Protein coupled receptors
GTP	Guanosine Triphosphate
HBSS	Hank's Balanced Salt Solution
HEK-293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KO	Knock Out
mAb	Monoclonal Antibody
PBS	Phosphate Buffer Saline
PEI	Polyethylenimine
PKA	Protein Kinase A
PKC	Protein Kinase C
WT	Wilde Type

Abstract

Introduction

Chemokine receptors belong to the family of G protein-coupled receptors. To date, the chemokine system is made up by 50 ligands and 21 receptors, classified on the basis of the cysteine pattern of the chemokines. CCR2 and its endogenous ligand CCL2 have been found to be involved in several diseases such as multiple sclerosis, rheumatoid arthritis, metabolic diseases, cancer and neuropathic pain. In these years different treatments have been proposed to inhibit the chemokine system from mAb to small molecules. Unfortunately, no cures are available on the market yet.

The aim of this project is to elucidate the binding site of two small molecule antagonists of CCR2 chemokine receptor: V-10-5191 and V-10-5299. The present project was fully planned and carried out in the laboratories of the Division of Medicinal Chemistry, Leiden/Amsterdam centre for Drug Research (LACDR), Leiden University (The Netherlands).

Methods

Site-directed mutagenesis and two different techniques of transfections: calcium phosphate and PEI transfection in HEK-293 cells were performed to express the receptor in study. Radioligand binding assays were applied to investigate the binding site of the molecules.

Results

Between the two transfection techniques evaluated only the polycation PEI transfection method yielded CCR2 expression for which ^{125}I-CCL2 binding could be detected. Subsequently the mutants Y49A$^{1.39}$, W98A$^{2.60}$, Y120A$^{3.32}$, H121A$^{3.33}$, F125A$^{3.37}$, I208A$^{5.44}$, I263A$^{6.55}$, E291A$^{7.39}$, E291Q$^{7.39}$, T292V$^{7.40}$ were evaluated using ^{125}I-CCL2 and the tritium derivatives of two small molecules [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299. Mutants Y49A$^{1.39}$, W98A$^{2.60}$, Y120A$^{3.32}$, E291A$^{7.39}$ were found to be important for ^{125}I-CCL2 binding. For [^{3}H]-V-10-5191 the mutants Y49A$^{1.39}$, W98A$^{2.60}$ and T292V$^{7.40}$ showed a significant decrease in specific binding followed by residues Y120A$^{3.32}$, E291A$^{7.39}$ and E291Q$^{7.39}$. Because of the large standard deviation no certain binding mode can be evaluated for the [^{3}H]-V-10-5299. However binding of [^{3}H]-V-10-5299 seemed to be less affected by any of the mutations in comparison to [^{3}H]-V-10-5191.

Conclusion

A better understanding of the binding site of antagonist of chemokine receptor can improve the knowledge in the pharmacological profile of the molecules in study and also give suggestions for the synthesis of further antagonist of CCR2 chemokine receptor.

Introduction

Chapter 1

GPCRs

1.1. An overview of proteins as receptors

The cells of our body can be compared with the worldwide human population; to understand each other they need to communicate. Among all the characters, the protagonists that play a crucial role in this communication system are the receptors. The receptors are proteins, localized on the surface of the cell membrane, across the cell membrane or in the cytosol. They are able to perform their task only after the interaction with a chemical messenger, which could be another protein, a hormone and also a small molecule as a drug. The chemical messenger is called ligand and can bind in several ways. The exact position in which this interaction takes place is known as *binding site* while the general area is known as *binding region* [2].

The interaction between these two parts triggers a cascade of events that leads to a series of biological effects inside the cells. There are some ligands soluble in lipids, such as steroid hormones and non-steroid hormones. They are able to diffuse across the membrane, bind the cytosolic receptor, form a complex ligand-receptor and go into the cell nucleus where they modulate DNA transcription. However, most of the ligands such as amines, amino acids, peptide and proteins are

soluble in water. Because of their hydrophilic nature, they are not able to diffuse across the cell membrane and therefore they bind to the extracellular site of a transmembrane receptor. These ligands are also called primary messenger, because they evoke the activation of an intracellular site of the receptor that interacts with another protein. This protein stimulates the release of a second messenger, which promotes the amplification of cellular signal and leads towards the final response. One family of receptors that follows this mechanism of action is the family of G-protein coupled receptors (GPCRs). This is the largest, the most ubiquitous and the most versatile family of membrane receptors, which is also the most common target of therapeutic drug [3]. The next paragraph is focussed on the GPCR classification, structure and functional characteristics.

1.2. GPCRs: classification and structure

GPCRs represent a productive area for drug discovery. As reported in literature, chemical programs to target GPCRs accounted for 40% of the portfolio of the pharmaceutical industry, with nearly 40% of these in clinical development [4]. It has been estimated that the human genome encodes more than 1000 GPCRs [5]. Structural studies revealed that GPCRs, as all transmembrane receptors, have three distinct domains [5]:

- an extracellular domain
- a transmembrane domain
- an intracellular domain

These different regions prove the amphipathic nature of this class of receptors. The hydrophilic extracellular and intracellular domains, containing polar and ionic side chain amino acids, can interact with the aqueous environment. The transmembrane domain, containing non-polar amino acids whit a α-helix tertiary structure, crosses the double phospholipidic stratus. GPCRs are members of the superfamily of the 7TM receptors and can be recognised from the precise number of transmembrane regions. The features that reveal a general common tertiary structure (Fig. 1) are: N-terminus domain, three loops in the extracellular region, C-terminus domain, three loops in the intracellular region and seven transmembrane regions with an α-helix structures [5].

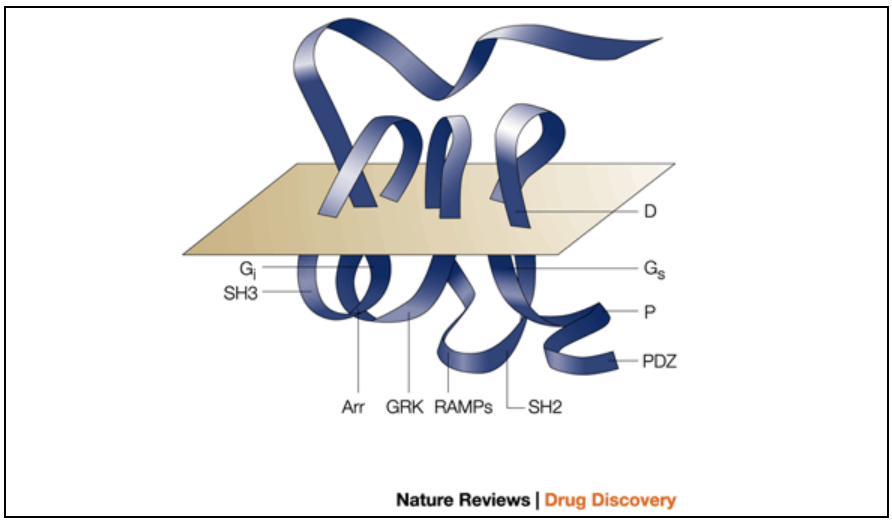

Fig. 1 Structure of 7TM GPCRs [3].

There are extensive amino acid sequence similarities that divide GPCRs into different classes, each with characteristic highly conserved residues, which define similar motifs. For example, in the large class of the rhodopsin receptor e.g. we have the DRY motif at

the cytoplasmatic end of the third transmembrane domain and prolines at the specific positions in helices 5, 6, 7 [5].

According to the Receptor Nomenclature and Drug Classification provided by the International Union of Pharmacology Committee, the superfamily of GPCRs is divided in different families or classes on the basis of their similarity. The main are: A, B, C or classes I, II, III respectively [6].

Each family generally shares over 25% sequence identity in the transmembrane core region, and a different set of highly conserved residues and motifs [5].

Family A (Class I) is by far the largest group and includes rhodopsin receptor, histaminic, adrenergic, dopaminergic and muscarinic receptors, the olfactory receptors. These receptors are characterized by the presence of cystein esterificated with the palmitic group at the C-terminus tail [5].

Family B (Class II) contains about 25 members including the gastrointestinal peptide hormone family, corticotropin-releasing hormone family, calcitonin and parathyroid hormone family. A long tail, including six conserved cysteins connected by disulfure bond, characterizes the N-terminus domain and is also involved in ligand binding. Receptors of this family seem to be mainly coupled with a G protein having a G_s subunit [5].

Family C (Class III) is quite small and includes the metabotropic glutamate receptor family, GABA receptor and the calcium sensing receptor. They are characterized by large extracellular amino terminus domain important for ligand binding and activation [5].

Endogenous ligands have been also identified for approximately 210

GPCRs, but for almost 100 receptors they have been not proposed yet. For this reason scientific community calls them “orphan receptor”, which represent an attractive field for pharmaceutical industry as target for future drug discovery [7].

1.3. GPCRs: receptor activation and signalling

The classical 7TM receptor signalling explains as in absence of a ligand, 7TM receptors are in a low–affinity state. After ligand binding, a transient high affinity tertiary complex of ligand, activate receptor and G protein is formed. G proteins are a family of membrane-associated proteins, so called because they bind GDP and GTP. It has been found that they are heterotrimeric proteins, containing a α subunit responsible for GDP and GTP binding and a, β and γ subunit, associated in a dimeric $\beta\gamma$ complex. The α subunit has intrinsic GTPase activity and is therefore able to hydrolyse GTP. After the binding of the ligand to the receptor, a series of conformational changes takes place and the high affinity ternary complex mentioned above is formed (Fig. 2). GDP is released from the G protein and is replaced by GTP. This leads to dissociation of the G-protein complex into α subunit and $\beta\gamma$ dimers, which both activate several effectors. Hydrolysis of GTP to GDP, a process regulated by RGS (regulator of G – protein signalling), leads to the reassociation of the three subunits and the end of the activation cycle. In order to improve this understanding step by step, but also to verify if a receptor is still functional or not, this mechanism has been investigated in several ways. Among the different assays the [^{35}S]-GTPγS functional assay, is one of the most widely used [7].

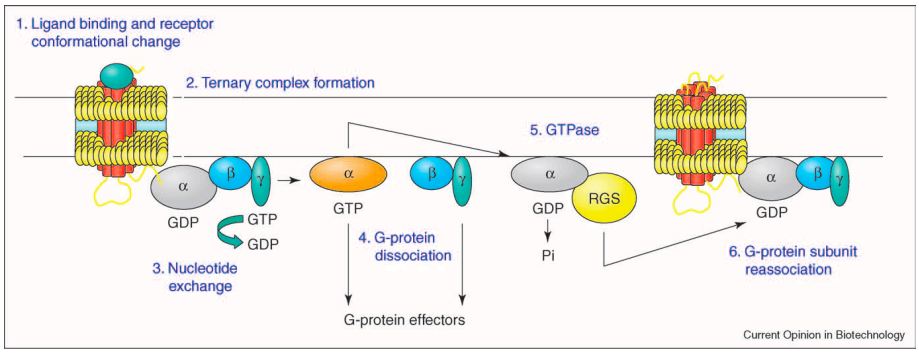

Fig. 2 GPCRs activation mechanism [7].

Detailed studies revealed that G proteins are extremely complex. To date each of these subunits is known to be a member of a gene family. It has been identified twenty different α subunits, six β subunits and twelve γ subunits. G- proteins are generally classified on the basis of their α subunits. Among all of them, the main subfamilies are: G_s proteins coupled to stimulation of adenylyl cyclase; G_i that provokes inhibition of adenylyl cyclase and the activation of some potassium channel such as GIRK (G protein coupled inwardly rectifying potassium channel); Gq coupled to the activation of phospholipase Cβ that provokes the release of DAG and IP3 as second messengers; G_o which reduces the probability of the activation of some voltage dependent Ca^{2+} channel involved in neurotransmitters release and $G_{12/13}$ coupled to the activation of RhoGEF (Guanine-nucleotide exchange factor) (Fig. 3) [7].

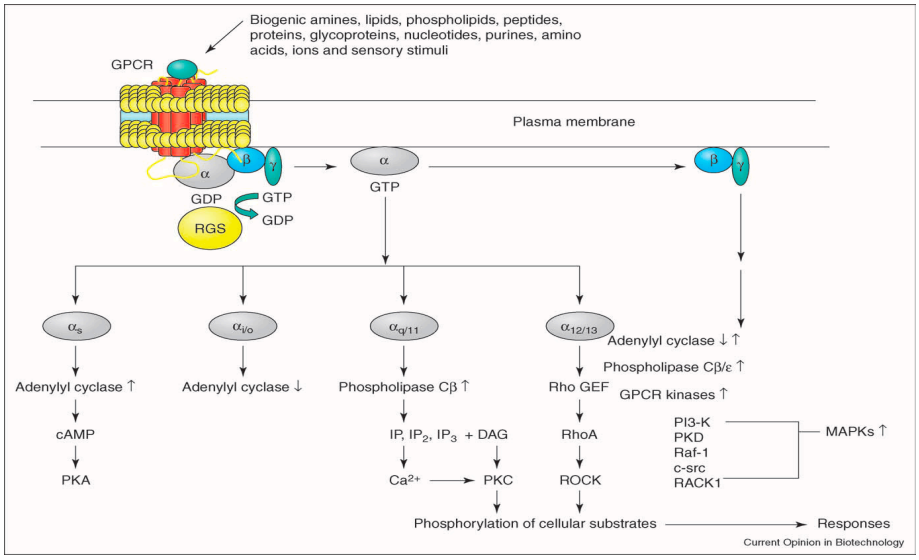

Fig. 3 GPCRs signaling pathways [7]

As documented by several studies GPCR signalling extended far beyond the traditional model of: "binding of agonist to the receptor → G protein activation → effector". It can also be regulated by desensitization and internalization, a process that involves phosphorylation, complex formation with the β-arrestin protein, recruitment of the resultant complex to clathrin-coated pits, endocytosis and at the end recycling to the membrane or lysosomal degradation. Receptor desensitization is a process that protects the cells against both the acute and the chronic overstimulation by the agonist. This mechanism operates at the level of the receptor, mediated by kinases (such as PKA and PKC) that provoke the uncoupling of G protein, but also at the level of the G protein [8].

The family of proteins RGS (regulator to G protein signalling) increases the rate of GTP hydrolysis bound to both G_i and G_q α-subunits, as reported for the protein RGS12 on CXCR2 [8].

Phosphorilation can be mediated by GRKs, a family of kinase having seven serin and threonin residues. However, while the first phosphorilation mechanism occurs also on non-activated receptor, the second mechanism acts only on the agonist-activated-GPCRs. GRKs phosphorilation is important for receptor internalization. In fact it leads to the recruitment of β-arrestin to the receptor and targets the receptor-β-arrestin complex to clathrin-coated pits. When internalization takes place, the receptor is incorporated in an endosome. Here it can have different destinies: one is the dephosphorylation and the recycling to the cell surface and another one is the degradation in lysosomes that leads to a process known as down regulation. It seems that β-arrestin also regulates 7TM receptor trafficking. Recent evidence indicates that β-arrestin internalization is necessary for down regulation, mediated by the binding of the β-arrestin lysine residues to single or multiple ubiquitins. This process, known as ubiquitination, directs the target protein to a proteosome for their destruction. Likewise, for the CXCR4 chemokine receptor, this process is important for the lysosomal sorting [9] [10].

The involvement of all these proteins and many others adaptor/scaffolding proteins that leads to the activation of several pathways, such as the ERK/MAPK cascade, show us the complexity and the compartmentalization of GPCRs signalling. The time frames of all these processes extend from seconds (phosphorylation), minutes (endocytosis) to hours (down regulation) [8].

This could also be important if we consider that the number of receptors may change over time such as in the course of a therapeutic treatment resulting in a down regulation, but also in some pathologic

conditions (neurodegenerative or inflammatory diseases) resulting in an up regulation. This is the case for the chemokine receptor.

Chapter 2

Structure, function, and inhibition of chemokines

2.1. The Chemokine System

Chemokines (Greek – kinos movement) or *chemo*tactic cyto*kines* are a large family of small soluble proteins (approximately 8-12kDa in size), which play a fundamental role in human physiology from embriogenesis to inflammatory response. They are released by leukocytes and their main role is chemotaxis, characterized by a concentration gradient that leads immune cells such as leukocytes, monocytes and neutrophilis towards the target tissue [11].

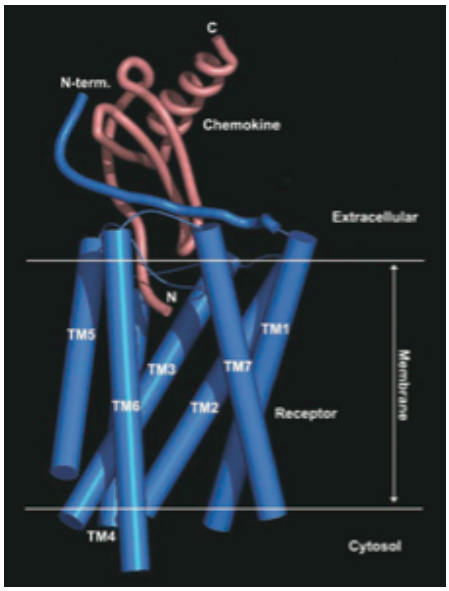

Fig. 4 A model of a hypothetical interaction between a chemokine (pink) and his receptor (blue) based on the solved structured of chemokine and bovine rhodopsine [11].

Based on their functionality, chemokines are classified as homeostatic chemokines constitutively expressed and inflammatory chemokines released after a pro-inflammatory stimulus (e.g. bacterial and virus infection or physical damage) by circulating leukocyte. It has been found that some of them also play a role in development, such as the development of the central nervous system, cardiogenesis and angiogenesis [12].

The structures of several chemokines have been solved by NMR and X-ray crystallography. Despite of their variable sequence homology, from less than 20% to over 90%, they have similar common features. Some conserved residues are important for their typical tertiary structure characterized by: a N-terminus of 6-10 amino acids, an N-loop ending with a single turn helix called 3_{10}-helix, a three stranded β-sheet and a C-terminal helix. After translation some chemokines are secreted from the cells while others remains bound to the cell surface. Chemokine classification is based on the pattern of cystein residues in the ligands. Most of them have four cysteins that form one disulfide bound between the first and the third cystein and a second one between the second and the fourth. These covalently bound cysteins give a typical Greek key structure to the ligands. According to the system of nomenclature introduced to identify chemokines and their receptors, there are four subfamilies: C, CC, CXC and CX3C (C represents cystein and X_n the number of noncystein residues). To date more than 50 chemokines and 21 chemokine receptors have been identified. It has been found that several chemokines can interact with different chemokine receptors, and one receptor is able to bind several different chemokines. For this reason chemokine system was

described as redundant, but this promiscuity can suggest the complexity and the subtlety of the chemokine system [11].

A general mechanism of leukocyte recruitment after a tissue injury or an infection consist of:

1. local up-regulation of pro-inflammatory chemokines in response to a signalling molecule such as TNF-α or IFN-γ;
2. binding of the chemokine to GAGs in the endothelian cell surface as a mechanism of retention at the inflammatory site [13];
3. binding of the chemokine to the receptor expressed on leukocyte cell surface;
4. activation of leukocyte receptor, increase in adhesiveness, extravasation of leukocytes from the blood;
5. migration of leukocyte towards chemokine gradients to the site of injury or infection.

Interaction chemokine-receptor has been described previously [14] dealing with a two-site model. It is hypothesized that the chemokine globular core binds to the extracellular N-terminus domain and the extracellular loops of the receptor. Otherwise the first residues of the N-loop of the ligand activate the receptor binding in the TM regions and some extracellular loops of the receptor (e.g. the DRY motif in the second extracellular loop) (Fig. 4).

The chemokine receptor is the largest family of G-protein coupled receptor and they share all the structural and functional features of this protein superfamily, widely discussed in the previous chapter.

However, since the determination of the first GPCR crystal structure of bovine rhodopsin [15] much progress has been made in the knowledge of chemokine/ligands-receptor interaction or more in general for GPCRs. It was followed by the first structures of liganded GPCRs for the β-adrenoceptors and adenosine A_{2A} receptor [16] [17] [18] and recently the CXCR4 chemokine receptor crystal structure was also solved [1], showing several differences to previous solved GPCRs. This has been revealed very important for mutagenesis and alignment study of chemokine receptor (Appendix) giving a wide impetus in chemokine drug discovery.

2.2. CCR2 chemokine receptor

Chemokine Receptor 2 represents a high–affinity receptor for the endogenous ligand MCP-1 (monocyte chemoattractant protein-1), cloned in the 1994 by Charo *et al*. [19] and also recognised as CCL2. It is the most characterized CCR2 ligand, but other member of macrophage chemotactive protein family of the chemokine (MCPclass), such as CCL8 (MCP2), CCL7 (MCP3), CCL13 (MCP4) bind to the same receptor [20]. The CCR2 receptor is present in mononuclear cells, basophils, memory T-cells, immature dentritic cells and Natural Killer cells. The alternative splicing of the carboxylic-terminal tail of gene encoding CCR2 generate two types of CCR2 receptors: type A CCR2a and type B CCR2b [19]. Further studies by Wong and co-workers showed that CCR2a is predominantly localized in the cytoplasm whereas CCR2b is exposed to cell surface [21]. It can activate the subunits $G_{\alpha i}$ and G_q of the trimeric G-proteins. The CCL2-CCR2 axis has been found to be

involved in several diseases: autoimmune diseases such as rheumatoid arthritis [22] and multiple sclerosis [23], metabolic diseases such as obesity and type II diabetes [24], respiratory diseases such asthma, allergic rhinitis [25], but also in cancer [26]. Recently it has been found that CCR2 has an important role in the neuropathic pain [27] and our attention is focused on this disease.

2.3. Neuropathic pain

Pain is a physiologic condition that occurs in response to adverse stimuli, but sometimes can evolve in pathological conditions due to damages to the nervous system, diabetes, cancer, toxic effects of drugs and infectious agent. These events can provoke an incorrect message on the nociceptive signal, leading towards a development of a spontaneous sensation of chronic pain, and sometimes excessive compared to the original stimulus (allodynia). The mechanism in which the chronic pain occurs shows first an up-regulation of the CCL2 by cell types of the peripheral nervous system such as, Schwann cells, resident macrophages, endoneurial fibroblasts and primary afferents of the dorsal root ganglia (DRG). The CCR2 signalling in DRG in neurons is exicitatory and therefore pronociceptive. Then, DRG neurons may transport CCL2 to central nerves ending of the spinal cord where it is released. Once here CCL2 can activate secondary neurons and some leukocytes expressing CCR2 [27].

It has been found that administration of exogenous CCL2 in spinal neurons can inhibit the activation of ionic current generated by stimulation of GABAa receptor [28]. Neuromodulation of GABAergic

inhibition could have facilitation in nociceptive transmission [29]. This means that CCL2 is a modulator of the nociceptive information in both peripheral and central nervous system. Knockout mice deficient in CCR2 receptor have also supported the latter mechanism and the role of CCL2/CCR2 axis in neuropathic pain. Testing of acute pain behaviour in CCR2 KO mice does not differ from WT mice. CCR2 KO mice did not show mechanical allodynia induced by partial ligation of the sciatic nerve, a model known induced hypernociception, or intraplantar administration of CCL2 [30]. Whereas overexpression of glial CCL2 show enhanced pain sensitivity [31]. White *et al*. [27] examined the possibilities that DRG could be the site of CCL2 action. Using two models of neuropathic pain: ligation of sciatic nerve and chronic compression of the DRG (CCD), an overexpression of CCL2 was provoked, suggesting that sensitive neurons themselves might be the site of chemokine receptor activation in the generation of chronic pain [32]. There are many factors involved in the pathophysiology of neuropathic pain but these findings showed how an intervention on CCL2/CCR2 axis could be important in the therapeutic treatment.

2.4. Therapeutic treatment of chemokine-involved diseases

Several inhibitors of the chemokine system have been developed with different biological and chemical profiles. The inhibitors investigated extended from monoclonal antibody, natural proteins encoded by viral genomes, gene therapy, antisense inhibitors, to small molecule binding to the target receptor [12]. For CCR2 a monoclonal antibody

MLN1202 has been developed by Millennium Pharmaceuticals. It has been in clinical trials for several diseases such as: metastatic cancer, atherosclerosis cardiovascular diseases and multiple sclerosis, but was not successful. mAb-derived therapeutics seems to be difficult to apply. The limited availability of GPCRs as purified proteins, and their low immunogenicity as TM proteins make arduous the generation of antibody able to recognise the GPCRs epitope [33]. More successful has been the research in small molecule antagonist of chemokine receptors. Indeed our attention will be focused on them.

2.5. Binding mode of small molecule antagonist of chemokine receptors

Surgand and co-workers [34] identified two binding pockets in the TM helices of the receptor: a minor and major binding pocket localized between TM1, 2, 3, 7 and TM3, 4, 5, 6 respectively. Moreover, Andrew *et al*. [35] generated a chimera receptor of CCR4-CCR5 showing how an intracellular binding site, localized in the helix 8, is important for the binding of aryl sulphonamides designed by Glaxo. As Kenakin reported [36]: GPCRs signalling is allosteric by nature because the endogenous agonist and the G proteins act as mutual allosteric modulators. However also small molecule can interact in this system as both in orthosteric and allosteric sites [37] [38] and they can show a competitive/surmountable or non-competitive/insurmountable behaviour [39]. As known, orthosteric sites (from Greek “ortho” means normal, correct or straight) binds to the endogenous ligand; whereas, the allosteric site is different from the active site (from Greek “allo” means other), and his activation can

generate conformational changes and alters important parameters such as affinity and efficacy of the receptor. Several pharmaceutical companies such as Merck, Johnson & Johnson, Glaxo, Novartis, Roche, Chemocentryx, Incyte/Pfizer showed their interest and invested in the field of CCR2 antagonists. Some of their compounds reached clinical trials phase II: such as MK-0812 and MLN1202 synthesized by Merck for atherosclerosis, MS and cancer, BMS-741672 by Bristol Myers Squibb for neuropathic pain, JNJ17166864 by Johnson & Johnson for allergic rhinitis and PF-04136309 by Incyte/Pfizer for pain. Despite these molecules showed a satisfactory outcome, other showed a lack in term of efficacy. One of the crucial points in development of CCR2 antagonists is the limited sequence homology (about 85%, Appendix), between murine CCR2 and human CCR2. Johnson & Johnson have reported large shift in potency for some of their compound between human and murine receptors [40]. This aspect can represent an obstacle in the evaluation of the CCR2 antagonists in preclinical rodent models. However Incyte developed a molecule INCB3344 or V-10-5191 having a good affinity on both human and mouse receptor, with a potency of 10nM to inhibit ^{125}I-JE (JE = murine CCL2) [41] and an IC_{50} (half maximal inhibitory concentration) of 7nM for hCCR2b determined with binding assays by Shin *et al,* 2009 [42]. The same molecule showed an *in vivo* inhibition or macrophage recruitment in mouse model of DTH and EAE and rat model of adjuvant arthritis [43]. The pharmacological profile of the CCR2 antagonist described ranges from a competitive such as for INCB3344 [44] to non-competitive behaviour such as for JNJ-141491 (J&J) [45]. However the most advisable manner is an insurmountable antagonism instead of a surmountable antagonism.

This is because the effect of the antagonist will be not overcome by higher doses of agonist from the receptor, leading towards better therapeutic aims [40]. Some features that characterize the interaction ligand-receptor are important to reach this purpose: allosteric interaction, non-competitive behaviour and a long receptor dissociation time also recognised as residence time.

2.6. Mutagenesis studies

To better understand the way in which a ligand binds to his receptor is to modify amino acidic residues and analyse the effects that the translated protein provokes, a process also recognised as mutagenesis. In site-directed mutagenesis single genes are engineered to produce mutant proteins. The site-directed approach may be done in such technique as alanine-scanning mutagenesis whereby residues are systematically mutated to alanine in order to identify residues important to the structure or function of a protein. Thanks to the help of a receptor homology model, it is also possible to rationalize the results and improve the next development in the discovery of new molecules with a better pharmacological outline. Otherwise some mutations can occur naturally in human genomic pattern and provoke pathological states. They are known as single nucleotide polymorphisms (SNPs). Considering CCR2, Bektas *et al*. found that G allele of CCL2 and 64I allele of CCR2 may be risk factors for oral squamous cell carcinoma [46]. Lin HL *et al*. also showed that one A allele of the V64I CCR2 gene polymorphism increased risk of cardiovascular diseases [47]. Many others SNPs have been found to represent risks in diseases such as hepatocellular carcinoma [48];

preeclampsia, a condition that can complicate pregnancy [49]; cervical cancer [50], etc. These list of SNPs are just an example that suggest how analysis in natural mutations could also help in a better understanding of CCL2-1/CCR2 axis, but also improve the knowledge of ligand-receptor interactions leading towards the design of more efficient drugs.

Aim of the project

The present project was fully planned and carried out in the laboratories of the Division of Medicinal Chemistry, Leiden/Amsterdam centre for Drug Research (LACDR), Leiden University (The Netherlands).

The study is focused on two small molecule antagonists of hCCR2b: INCB3344 or V-10-5191 [44] and V-10-5299 [51]. Ten mutants of hCCR2 (Y49A$^{1.39}$, W98A$^{2.60}$, Y120A$^{3.32}$, H121A$^{3.33}$, F125A$^{3.37}$, I208A$^{5.44}$, I263A$^{6.55}$, E291A$^{7.39}$, E291Q$^{7.39}$, T292V$^{7.40}$) were kindly provided by J. Pease and will be used to get insight into the binding sites of V-10-5191 and V-10-5299. These mutants have been predicted by computational modelling by Hall *et al*. [52] to be involved in the binding of dual antagonists of human CCR5/CCR2: TAK-779 (N,N-dimethyl-N-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5Hbenzohepten-8-yl] carbonyl]amino]benzyl]tetrahydro-2H-pyran-4-aminium) and Teijin compound 1 (N-(carbamoylmethyl)-3-trifluoromethyl-benzamido-parachlorobenzyl 3-aminopyrrolidine). Comparison between two different techniques of transfection in mammalian HEK-293 cells: calcium phosphate and PEI transfection, will lead to the better expression of the receptor. The expression of the receptor will be checked using ^{125}I-CCL2, [^{3}H]-V10-5191 and [^{3}H]-V-10-5299 in radioligand binding assays. The analysis of the effects of these mutants on specific binding will add information on the chemical interactions that occurs between the residues involved in the

binding site and the three molecules. These data could support further studies in the design of new antagonists of chemokine receptor CCR2, improving the knowledge on the binding site of GPCRs and clarify the pharmacological profile of the molecules already known.

Chapter 3

Materials and methods

3.1. Chemical and reagents

The CCR2-specific antagonists, ligand V-10-5191 also recognized as INCB3344 and V-10-5299 were kindly provided by Vertex Pharmaceutical incorporated (San Diego, CA). The unlabelled endogenous ligand CCL2 was purchased from PeproTech (Rocky Hill, NJ). The radiolabeled tritium compounds [^{3}H]-V-10-5191 (specific activity 32 Ci mmol^{-1}) and [^{3}H]-V-10-5299 (specific activity 64 Ci mmol^{-1}) were kindly provided by ViTrax (Placentia, CA), and the iodinate endogenous radioligand ^{125}I-CCL2 (specific activity 2200 Ci mmol^{-1}) was provided by Perkin-Elmer (Waltham, MA). Poly-D-Lysine was provided by Invitrogen. The monoclonal HA-tag EPITOPE antibody was purchased by Novels Biologicals (Cambridge, UK) and the secondary antibody conjugated goat anti-rabbit by Novels Biologicals (Cambridge, UK). Linear PEI (MW 25000Da) was purchased from Polysciences, Inc. Albumin standard and BCA Reagents were purchased from THERMO Scientific, USA. All other reagents were of the highest purity available and were obtained from standard commercial sources.

3.2. DNA constructs

The cDNA encoding human chemokine (C-C motif) receptor 2 (CCR2) WT and mutants (Y49A$^{1.39}$, W98A$^{2.60}$, Y120A$^{3.32}$, H121A$^{3.33}$, F125A$^{3.37}$, I208A$^{5.44}$, I263A$^{6.55}$, E291A$^{7.39}$, E291Q$^{7.39}$, T292V$^{7.40}$) having a sequence of 3xHA-tag encoded at the N-terminus tail were subcloned into pcDNA3.1 as a vector containing ampicillin resistence gene and kindly provided by J. Pease (http://www.cdna.org). WT CCR2 lacking the HA-tag was kindly provided by VU University (M. Smit). All constructs were verified by DNA sequencing, using program BLASTIN 2.2.26 + BaseClear, before use.

3.3. Transformation of competent cells: E. coli DH5α

In order to produce large amounts of identical DNA, plasmid DNA having the constructs on study were introduced in prokaryotic cells: E. coli DH5α stored in 20% of glycerol at -80°C (purchased by Invitrogen). A Heatshock at 42°C was used in order to make competent bacterial cells. Transformed bacteria were plated on a culture plate (10cm of diameter) in LB agar medium containing ampicillin 250mg/mL to select the transformed colonies. After incubation overnight at 37°C the plates were stored at 4°C.

3.4. Isolation of plasmid DNA using Qiagen® Midi Kit

A single colony from the selective plates was isolated and inoculated in 50mL of LB medium containing ampicillin 250mg/mL as selective antibiotic. The incubation was carried out overnight at 37°C in water bath with vigorous shaking. The bacterial cells were harvested by

centrifugation and the pellet was resuspended in buffer P1 containing 50mM Tris·Cl, pH=8.0; 2–8°C, 10mM EDTA; 100μg/ml RNase A, then treated whit a lysis buffer P2 containing 200mM NaOH, 1% SDS (w/v) 15–25°C. Neutralization buffer P3 was added (3.0M potassium acetate, pH=5.5 15–25°C or 2–8°C) in order to promote precipitation of genomic DNA, proteins and cell debrids. After 2 steps of centrifugation at 9384xg and one filtration with miracloth, the supernatant was applied to an equilibrate Qiagen-tip using equilibration buffer (750mM NaCl; 15–25°C 50mM MOPS, pH=7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)) and allowed it to empty. The tip was washed once with wash buffer (750mM NaCl; 15–25°C 50mM MOPS, pH=7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)) and the DNA eluted with Elution Buffer (1.25M NaCl; 15–25°C 50mM Tris·Cl, pH=8.5; 15% isopropanol (v/v)). The DNA was precipitated, first with isopropanol and after centrifugation, with the more volatile 70% ethanol. The pellet of plasmid DNA was resuspended in MilliQ water.

3.5. Quality and concentration of plasmid DNA

DNA concentration was determined by UV-spectrophotometry at 260nm using an Eppendorf BioPhotometer. The ratio 260/280nm and 260/230nm were also measured to check contamination by protein and solvent, respectively. DNA purity was checked using the restriction enzyme Hind III provided by Invitrogen, in order to exclude genomic DNA contamination. All samples with enzyme treatment and without restriction enzyme were checked performing Agarose gel electrphoresis

containing 1% agarose, TAE buffer and Ethidium bromide for visualisation of DNA by UV-light using a Biorad detector.

3.6. Cell culture

HEK-293 cells, purchased from Invitrogen (Carlsbad, CA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of Newborn Bovine Serum (NBS), 50IU/mL of penicillin and 50µg/mL streptomycin. Cells were grown in a humidified incubator at 37°C with 7% of CO_2 and subcultured by trypsinization twice weekly.

3.7. Calcium phosphate transient transfection

One day before calcium phosphate transfection, HEK-293 cells were split (1:6) in order to have 50-60% of confluence the next day. A solution of sterile MilliQ water, $CaCl_2$ 1M, and plasmid DNA having the construct of interest was mixed in a falcon tube. After 1-2 minutes, HBBS (280nM NaCl, 10nM KCl, 1.5mM Na_2HPO_4, 50mM HEPES, pH=7.05) was added drop wise. After 1 minute 1mL of the DNA-mixture was added to the cells (10cm plate). Incubation at 37°C with 7% CO_2 was performed and the cell harvested 48 hours post-transfection.

3.8. Polycation polyethylenimine (PEI) transient transfection

HEK-293 cells were transiently transfected with the polycation molecule PEI. Different plasmid DNA concentrations were used: 1, 2.5 and 5µg to a total amount of 5, and 7.5µg and 10µg to a total volume of

10µg per 250µL of DNA. pcDNA 3.1 (-) plasmid was used as empty control plasmid DNA, lacking the construct of interest. A total DNA/PEI ratio of 1:6 was used. A sterile PEI solution of 1mg/mL and NaCl 150mM were prepared. A DNA mixture was used adding plasmid DNA to a total volume of 250µL NaCl 150mM. DNA and PEI solutions were mixed well and incubated for 20 minutes at room temperature (RT). Fresh medium was added to the cells and 500µL of transfection mix were added to the cells having 50-60% of confluence (1.4-1.8 million cells/10cm dish were seeded the day before). Transfected cells were collected after 48 hours.

3.9. ELISA: Enzyme Linked Immunoabsorbent Assay to check receptor expression

The Immunoabsorbent Assay was performed 48 hours after transfection. 24 hours after transfection a 96-well plate was treated for 30 minutes at RT with Poly-D-lysine 0.1mg/mL. 100000 cells/100µL in culture medium were seeded plate and incubated at 37°C, 7% CO_2 for 24 hours. After incubation the medium was removed and 100µL of primary antibody rabbit-HA-tag EPITOPE (1:5000 in DMEM) was added. After 30 minutes of incubation at 37°C, 7% CO_2 and a washing step, using DMEM/HEPES 25mM 100 µL, a solution of Goat-anti-Rabbit mAb used at 1:5000 dilution in empty DMEM was added. After that, the incubation step was carried out at 37 °C and 7% CO_2 for 30 minutes. The medium was removed and the plate was washed twice with 100µL PBS warmed at 37°C. The wells were dried for half an hour and incubated for 5 minutes in dark covered in aluminium foil after addition of 3,3',5,5'-Tetramethylbenzidine (TMB). The reaction

was stopped with 100µL of H_3PO_4 1M and the absorbance was measured at 450nM after 5 minutes using Victor ^{2}V plate reader from Perkin Elmer.

3.10. Membrane preparation from transient transfected HEK- 293 cell lines for binding studies

The recombinant HEK-293 cells expressing either wilde type hCCR2 and the mutants were harvested after 48 hours after transfection. The medium was aspirated and the cells were removed from the plates by scraping into 5mL of PBS. The membrane suspension was centrifuged at 700g for 5 minutes. The supernatant was discarded and the pellet was kept on ice. The pellet was homogenized in a volume (about 20mL for 30 plates) of 50mM Tris-HCl and 5mM $MgCl_2$, pH=7.4 with the Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The membrane suspension was centrifuged at 100000 x g in the Optima LE-80K ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA) with a Ti 70 rotor at 4°C for 20 minutes. The pellet was resuspended in 10mL of 50mM Tris-HCl and 5mM $MgCl_2$ buffer and the homogenization and centrifugation step was repeated. Finally, the pellet was resuspended in 50mM of Tris-HCl buffer and 5mM $MgCl_2$. Aliquots of 100 and 250µL were stored at -80°C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method [53].

3.11. Equilibrium radioligand binding assays

3.11.1. ^{125}I-CCL2 Binding Assays

Binding assays were performed in a 100µL reaction volume containing 50mM Tris-HCl (pH=7.4), 5mM $MgCl_2$, 0.1% CHAPS assay buffer, membranes at a concentration of 25µg. Nonspecific binding was determined with 100nM CCL2. The endogenous iodinate compound ^{125}I-CCL2 (K_d=0.068nM [54]) was used as radioligand in a range of concentration 0.1-0.23nM, between twice or fourth times the K_d value. Binding was allowed to proceed for 150 minutes at 37°C of incubation. Incubation was terminated by addition of 50mM Tris-HCl (pH=7.4), 5mM $MgCl_2$, 0.05% CHAPS, 0.5M NaCl as ice-cold wash-buffer. Separation of bound from free was performed under reduced pressure using a Brandel harvester, by rapid filtration through a GF/B filter precoated on 0.25% of PEI solution in 50mM Tris-HCl (pH=7.4), 5mM $MgCl_2$ in order to reduce non specific binding. Filters were washed three times with ice-cold wash-buffer and collected in γ-tubes. Scintillation fluid was not required for the iodinate radioligand and the filter-bound radioactivity was counted with Perkin-Elmer's Wallac Wizard 1470 automatic gamma counter (Perkin-Elmer). Each data point for nonspecific and specific binding was done in duplicate for the screening of each mutation and the wilde type

3.11.2. [^{3}H]-V-10-5191 Binding Assays

Binding on human CCR2 receptor was determined on membranes from HEK-293 cells expressing the human receptors, using [^{3}H]-V-10-5191 (K_d=5.00 nM [55]) as radioligand. Membranes containing 25

or 40µg of proteins were incubated in a total volume of 100µl of 50mM Tris-HCl (pH=7.4), 5mM $MgCl_2$, 0.1% CHAPS assay buffer and [^{3}H]-V-10-5191 (final concentration 12.2-16.5nM) for 2 hours at 25°C of incubation using a 96-well plate. Nonspecific binding was determined in the presence of 10µM V-10-5191. Incubation was terminated by rapid filtration over a 96-well GF/B filter plate under reduced pressure using a PerkinElmer Filtermate-harvester (PerkinElmer, Groningen, The Netherlands). Filters were washed ten times by addition of 50mM Tris-HCl (pH=7.4), 5mM $MgCl_2$, 0.05% CHAPS ice-cold buffer and 25µL of Microscint scintillation cocktail (PerkinElmer) was added to each well. After 2 hours of incubation, the filter- bound radioactivity was determined by scintillation spectrometry using P-E 1450 Microbeta Wallac Trilux liquid scintillation counter (PerkinElmer) by using uncoated 96-well GF/B filter plates. As described in the previous assay entitled "^{125}I-CCL2 Binding Assay", each data point was done in duplicate for the screening of each mutation.

3.11.3. [^{3}H]-V-10-5299 Binding Assays

Assay conditions were similar to those described for [^{3}H]-V-10-5191 binding assay. Nonspecific binding was determined using 10µM of the corresponding unlabelled compound V-10-5299. The radioligand binding was performed using [^{3}H]-V-10-5299 (K_d=5.8nM [54]) (final concentration 2.5-3nM). The experiment was terminated as described in the section entitled "[^{3}H]-V-10-5191 Binding Assay" using uncoated 96-well GF/B filter plates.

3.12. Data analysis

All data was analysed using Microsoft Office Excel 2003 and GraphPad Prism v.5 (GraphPad Software Inc., USA). They were expressed as the mean ± SEM. Data from the binding of radioligands from hCCR2 WT and mutants were reported as the mean ± SEM from three separates experiments.

Chapter 4

Results and discussion

4.1. Gene delivery system: comparison between two transfection techniques in HEK-293 cells for the hCCR2 chemokine receptor through binding assays

Two different transfection techniques were compared: the first one was calcium phosphate transfection and the second one was carried out using the polycation molecule PEI (25kDa). For both transfection methods, common conditions are crucial for a favourable outcome. Cells health, cells confluence at 50-60%, quality and quantity of plasmid DNA, medium, all of them are important parameters to perform a successful experiment [56]. Previous studies suggested that for calcium phosphate transfection, 10µg of plasmid DNA was an optimal amount to yield high receptor expression. However, binding of ^{125}I-CCL2 to the transfectants could not be detected. Therefore, in order to have a better expression of WT and mutant constructs of the hCCR2b receptor in this study another transfection method was included using polyethylenimine (PEI). The first PEI transfection was performed using 1µg, 2.5µg and 5µg of plasmid DNA. Receptor expression was checked using three different radioligands: the endogenous ligand ^{125}I-CCL2 (K_D=0.068nM [54]), and two small

molecules antagonist radioligands: [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299 (K_D=5.0nM and 5.8nM respectively, [54] [55]) (Fig. 5).

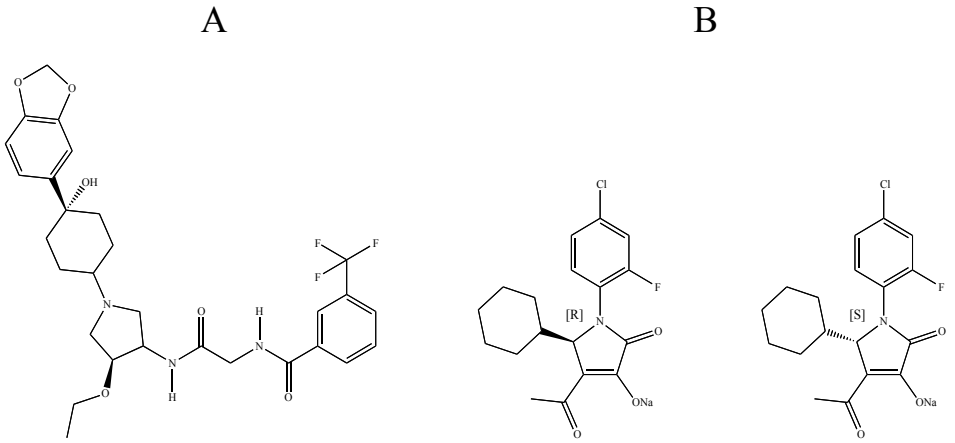

Fig. 5. Structure of small molecule antagonists of CCR2 Chemokine receptor: V-10-5191 also recognised as INCB3344 (A), V-10-5299: active (R)-4-Acetyl-1-(4-chloro-2-fluorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one and inactive (S)-4-Acetyl-1-(4-chloro-2-fluorophenyl)-5-cyclohexyl-3-hydroxy-1,5-dihydro-2H-pyrrol-2-one enantiomers (B).

Fig. 6 shows the specific binding of the three different molecules and gives an indication of a different efficiency in receptor expression. For calcium phosphate transfection, the tritium radioligands (blue and red bar in the graph) showed a high window in specific binding of hCCR2b. However, ^{125}I-CCL2 specific binding was not detected (green bar in Fig. 6). For PEI transfection high levels of specific binding of [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299 were also detected but differently from what observed with the previous experiment, ^{125}I-CCL2 specific binding was significantly measured. Furthermore, the data concerning PEI transfection revealed that an increase in the amount of plasmid DNA is related to higher receptor expression. The maximum amount of DNA for the calcium phosphate and PEI transfection was 10µg and 5µg respectively. These data suggested that

in our study PEI transfection seemed to be more efficiently in HEK293 cells.

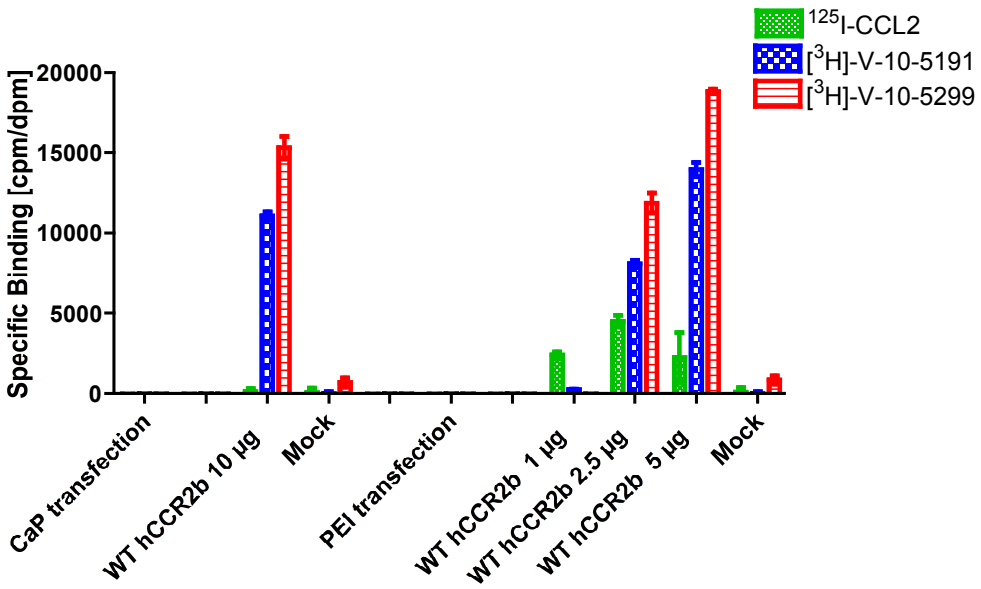

Fig. 6. Displacement from calcium phosphate and PEI transfected HEK-293 cells (40µg/25µL membranes). The picture shows the specific binding of three radioligands: ^{125}I-CCL2 0.15nM (green), [^{3}H]-V-10-5191 16nM (blue), [^{3}H]-V-10-5299 2.43nM (red). WT hCCR2 10µg is correlated to calcium phosphate transfection and WT hCCR2 1µg, 2.5µg and 5µg to PEI transfection. Mock is used as negative control.

4.2. Optimization of PEI transfection and evaluation through binding assays

A subsequent PEI transfection was performed on HEK-293 cells using a new WT hCCR2b DNA construct having a 3xHA-tag epitope at the N-terminus domain. In order to verify if also in this case, 5µg was the optimal concentration of plasmid DNA, the transfection was performed increasing the amount of plasmid DNA at 7.5µg and 10µg. The ratio DNA/PEI was maintained at 1:6. Data showed that 5µg was not the optimum concentration. In fact, using radioligand binding

assays higher levels of receptors expression were detected at 7.5µg and 10µg. Only for this transfection, an Enzyme linked immunoabsorbent assay (ELISA) was performed to check receptor expression (Fig. 7) using the appropriate kit as described in the section of "Material and methods".

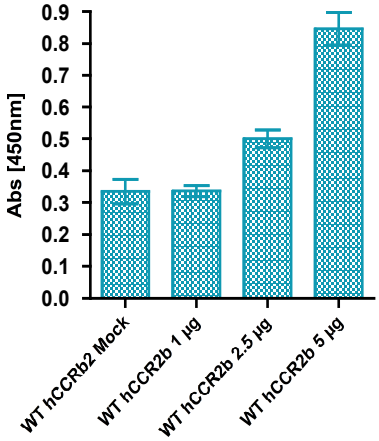

Fig. 7 ELISA from PEI transfected HEK-293 cells: absorbance detected at 450nm for mock (negative control), WT hCCR2b in HEK-293 cells transfected with 1µg, 2.5µg and 5µg of plasmid DNA.

Comparing the absorbance at 450nm of the cells transfected with the mock and the construct of 5 µg and 2.5µg an increase of 2.6 and 1.5 fold was observed respectively. In contrast, the cells transfected with 7.5µg and 10µg did not show a high percentage of life (checked with colorant Trypan Blue in a BioRad Reader) and this caused a detachment from the wells. This aspect did not allow performing ELISA for the cells transfected with higher concentration of plasmid DNA. However comparing the first PEI transfection with the second, performed with the HA-tag epitope WT hCCR2b, a decrease in the maximal signal was observed. This suggested that a change in plasmid

construct (typology and quality) might effect on intensity of receptor expression. Moreover, PEI transfection performed at 10µg was carried out using a doubling in PEI amount (ratio DNA/PEI = 1:6). Even if the cells expressed significantly the receptor, a treatment with a ratio PEI/DNA 1:6 using high DNA concentration, showed a decrease in growing. These data suggested that the PEI amount added could be toxic for health cells [57]. Although high levels of cell death occurred two days after transfection, the receptor was still highly expressed. In fact as Fig. 8 shows the most efficient expression was observed for the constructs of 7.5µg and 10µg. The ^{125}I-CCL2 manifested a proportional increase in specific binding reaching the highest window at 10µg. The tritium labeled antagonists [^{3}H]-V-10-5191 showed a decreased of 2.5-3 fold in specific binding compared to the endogenous ligand. The radioligand [^{3}H]-V-10-5299 showed the lowest signal with a decrease of more than 5 fold compared to ^{125}I-CCL2 but still considerable to detect the construct of 10µg as the most effective. Therefore, subsequent transfections of WT, mock pcDNA3.1(-) and the 10 mutants were carried out with 10µg of plasmid DNA.

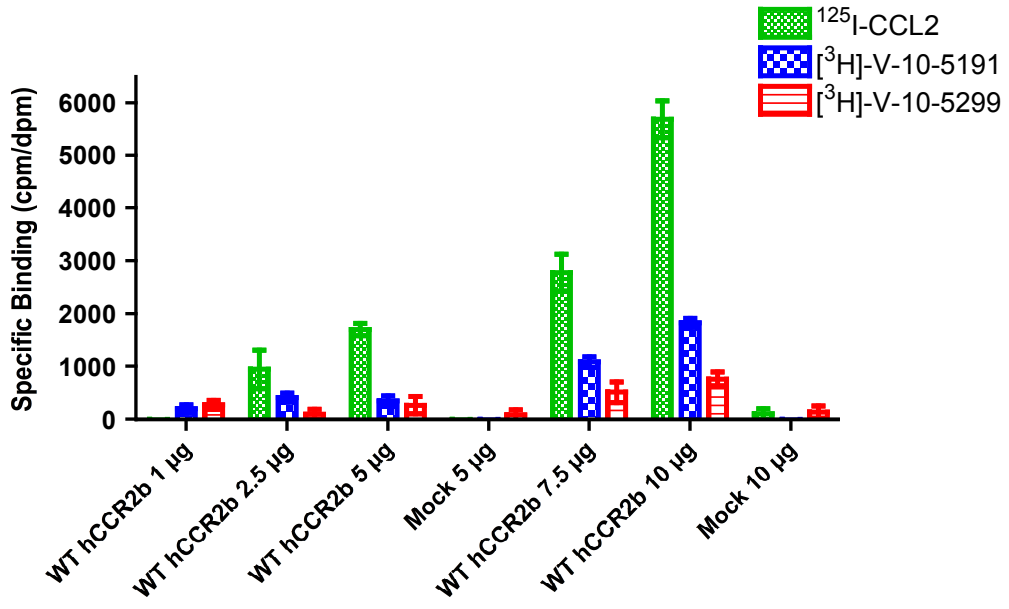

Fig. 8 Displacement from PEI transfected HEK cells (25µg/25µL membranes). The picture shows the specific binding of the three radioligand: [^{125}I]-CCL2 0.22nM (green), [^{3}H]-V10-5191 12nM (blue), [^{3}H]-V10-5299 3.15nM (red) on WT hCCR2 using different amount of DNA: 1, 2.5, 5, 7.5 and 10µg and mock as negative control.

4.3. Evaluation of hCCR2b mutants and WT expression through binding assay and identification of the binding site of V-10- 5191 and V-10-5299 on hCCR2

In this study we carried out the expression of the WT and ten mutants of hCCR2b, with a relevant aim not yet evaluated in literature: elucidate the binding site of two small molecule, non peptidergic ligands, antagonists of hCCR2 chemokine receptor: V-10-5191 recognised as INCB3344 [55] [58] and V-10-5299. As reported in literature, V-10-5191 or INC3344 was effective in lowering macrophage in target tissues and in suppressing development of disease models such as: mouse model of multiple sclerosis, rat model of inflammatory arthritis and mouse model of obesity [58]. Moreover,

the molecule exhibited IC_{50} of 5.1nM (hCCR2) and 9.5nM (mCCR2) in binding affinity and 3.8nM (hCCR2) and 7.8nM (mCCR2) in chemotaxis activity [58]. It also showed a moderate hERG binding activity, a good oral bioavailability and a promising pharmacokinetics profile in mouse [58]. Less study are present in literature for the molecule V-10-5299 but has been reported that the R-active enantiomer of the molecule used in a treatment of nerve-injured rats showed reversal of tactile hyperalgesia [51]. The present study focused his attention on the binding site of these two molecules using a mutagenesis approach. The effects of mutations on receptor expression have been evaluated with the use of three radiolabeled compounds: ^{125}I-CCL2, [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299 and described in the following sections.

4.3.1. Effect of hCCR2b mutations on binding of the labelled endogenous ligand ^{125}I-CCL2

The ability of each mutant to bind ^{125}I-CCL2 was examined and except for some mutants, a specific binding was detectable (Table 1) (Fig 9). For mutants Y49A$^{1.39}$, W98A$^{2.60}$, Y120A$^{3.32}$, E291A$^{7.39}$ the specific binding was significantly reduced, lower than 30% compared to WT. For F125A$^{3.37}$, I263A$^{6.55}$, E291Q$^{7.39}$ the signal was lower than 50%. Differently from what observed for other mutants, H121A$^{3.33}$ showed an increase in specific binding while for I208A$^{5.44}$ and T292V$^{7.40}$ no notable changes compared to WT were observed (Fig. 9). According to literature [59] these data showed that the upper part of TM1, TM2, TM3 and TM7 are important for the binding to the endogenous ligand. Mutations of the aromatic residues such as

Tyr49$^{1.39}$, Trp98A$^{2.60}$, Tyr120A$^{3.32}$ suggested that hydrophobic interactions or H-bond affect the binding of CCL2 to TM1, TM2 and TM3 of CCR2. Electrostatic interactions are more involved in the binding to TM7. Mikhail et al. [60] sustained that reversing the negative charge in Glu291$^{7.39}$ to a positive charge in lysine, ligand binding was completely abrogated. In this study the mutation of the charged side chain of the glutamic acid into the hydrophobic alanine caused a loss in specific binding more than the substitution to a polar glutamine. Other mutants did not showed a significant influence on the binding except for H121A$^{3.33}$. The loss of a positive charge of the hystidine seemed to stabilize the binding of the ligand to the receptor. These are in accordance with the data obtained by Hall *et al.* [52]. In fact, a decrease in IC_{50} of 2.27 fold compared to WT was described for H121A$^{3.33}$ and a displacement less than 50% was observed for Tyr49$^{1.39}$, Trp98A$^{2.60}$, Tyr120A$^{3.32}$ and Glu291$^{7.39}$. What mentioned above suggested that mutants H121A$^{3.33}$ increased the affinity of CCL2 for the receptor, whereas the seconds produced a decrease.

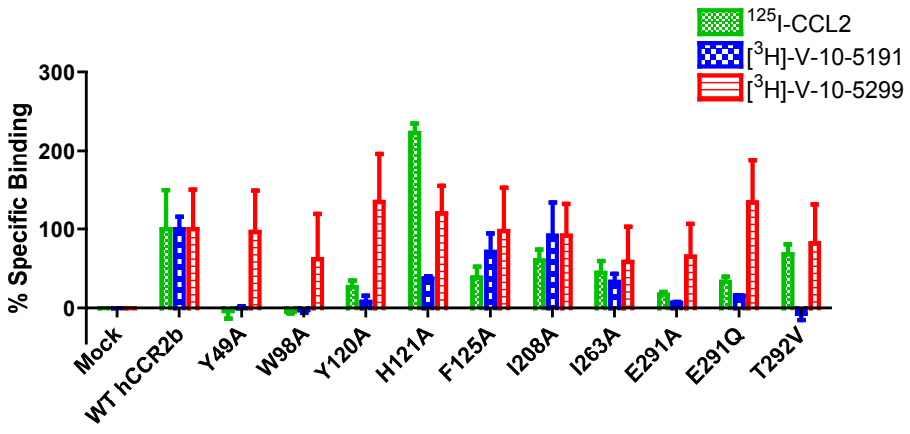

Fig. 9 Displacement from PEI transfected HEK cells. The picture shows the percentage of specific binding of the three radioligand: ^{125}I-CCL2 (green), [^{3}H]-V10-5191 (blue), [^{3}H]-V10-5299 (red) on mock (negative control), WT and 10 mutants of hCCR2b (using 10µg of plasmid DNA).

4.3.2. Effect of hCCR2 mutations on binding of labelled antagonists [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299

Fig. 9 shows that the two antagonists [^{3}H]-V-10-5191 and [^{3}H]-V-10-5299 have completely a different profile. The results of this experimental section are summarized in Tab. 1. The binding of the first ligand is considerable influenced. In fact, for mutants Y49A$^{1.39}$, W98A$^{2.60}$ and T292V$^{7.40}$ no specific binding was measured. Mutants Y120A$^{3.32}$, E291A$^{7.39}$ and E291Q$^{7.39}$ were also significantly effected showing a specific binding lower than 30%. Moreover H121A$^{3.33}$ and I263A$^{6.55}$ showed a decrease less than 50%. Conversely, mutants F125A$^{3.37}$ and I208A$^{5.44}$ did not produce a loss in binding or at least in a very negligible percentage, suggesting that they are localized outside the binding pocket. Finally, as reported by the red bar in Fig. 9, for the [^{3}H]-V-10-5299 ligand a high value of standard deviation was obtained and did not allow us to identify the influence of the mutants on specific binding.

4.3.3. Orthosteric or allosteric compound? ^{125}I-CCL2 and [^{3}H] labelled small antagonists binding in comparison

Divergence and similarities can be observed in the binding mode of [^{3}H]-V-10-5191 (blue bar in Fig. 9) compared to the iodinate CCL2. As for ^{125}I-CCL2, mutants Y49A$^{1.39}$, W98A$^{2.60}$ showed completely a lack in specific binding. The ability of Y120A$^{3.32}$, E291A$^{7.39}$ and E291Q$^{7.39}$ mutants to bind [^{3}H]-V-10-5191 was also reduced.

Differently from ^{125}I-CCL2, mutant T292V$^{7.40}$ affected clearly [^{3}H]-V-10-5191 binding and H121A$^{3.33}$ also lowered the specific binding of the tritium radioligand to the receptor. Data summarized in Tab. 1 shows for the molecule V-10-5191 a series of chemical interactions that cooperate strongly with these residues of interest.

Tab. 1 Summary of displacement of the three radioligands in use from mutants of CCR2: ↓↓↓ specific binding less than 30% compared to WT, ↓↓ specific binding less than 50% compared to WT, ↓ specific binding lower than WT, Nil negligible specific binding, mention ↑, - specific binding approximately as wild type.

Construct	^{125}I-CCL2	[^{3}H]-V-10-5191	[^{3}H]-V-10-5299
Y49A	↓↓↓	Nil	-
W98A	↓↓↓	Nil	-
Y120A	↓↓↓	↓↓↓	↑
H121A	↑	↓↓	↑
F125A	↓↓	↓	-
I208A	↓	-	-
I263A	↓↓	↓↓	↓
E291A	↓↓↓	↓↓↓	-
E291Q	↓↓	↓↓↓	↑
T292V	↓	Nil	-

They were further explored in a homology model of CCR2. The most relevant amino acids and binding interaction are represented in Fig. 10. The homology model suggested that residue Tyr49$^{1.39}$ could be involved in hydrogen bonds with the amide nitrogens (distance ~ 5Å) of the small molecule and could be stabilized with π-π interaction by the residue W98$^{2.60}$. Glu291$^{7.39}$ is known from literature to interact with the pyrrolidine nitrogen through electrostatic interactions [52].

Tyr120$^{3.32}$ forms an important interaction with the ethoxy group of the pyrrolidine ring. Other residues such as His121$^{3.33}$ that also showed a decrease in specific binding of [^{3}H]-V-10-5191 from hCCR2, could interact through van der Waals interactions. These data showed that the radioligands ^{125}I-CCL2 and [^{3}H]-V-10-5191 overlap some residues. They could share a common binding site on the receptor suggesting that molecule V-10-5191 could be an orthosteric antagonist of human chemokine receptor CCR2. For the molecule V-10-5299 it was difficult to detect the residues involved in the binding. However data showed for all the mutants, suggested that the two molecules have a different binding site. The size of the antagonists (~600Da) is smaller compared to the endogenous ligand (~8600Da) and several new mutations could affect the binding of the molecule to the receptor adding new information on the binding mode of the molecule that could be in the transmembrane region but also extracellular or intracellular. The sulfonamide antagonists of CCR4 receptor, e.g., synthetized by Glaxo and described by Andrew *et al.* [35], showed an intracellular binding site. This binding site is located close to the site of G protein-coupling to the receptor, and therefore the presence of antagonist at this site could prevent activation of G protein [54]. Recently, It has been demonstrated that the molecule V-10-5299 recognised as CCR2-RA-[R] has a similar binding site with the molecule JNJ-27141491, previously described [45], and they inhibit CCR2 with a similar mechanism [54]. Mutations localized in the intracellular region of hCCR2b might elucidate the hypotesis of an intracellular binding site for V-10-5299. For this reason in order to assert if the molecule is an orthosteric or allosteric antagonist further investigations are required.

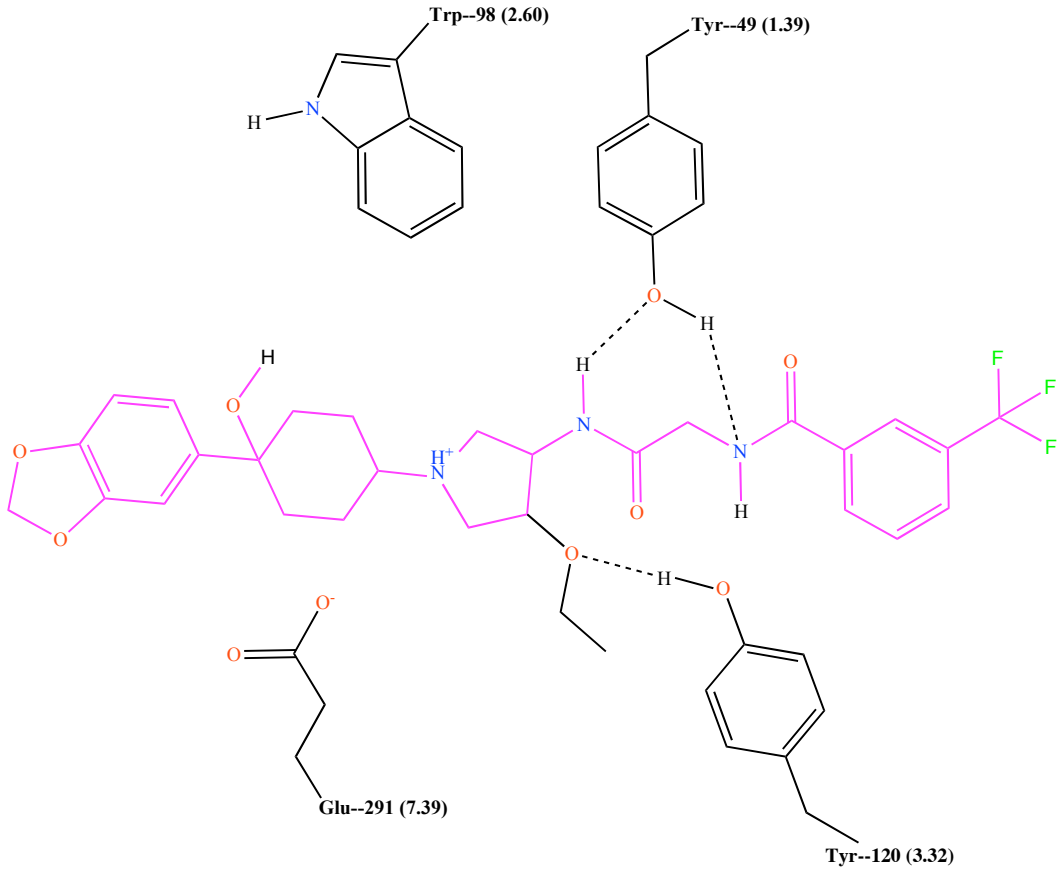

Fig. 10 Residues involved in the binding of V-10-5191 on CCR2 and binding interactions.

4.4. Comparison of the interhelical contacts between Teijin and V-10-5191 binding on hCCR2 receptor

In previous study reported by Hall *et al.* [52], the mutants analyzed in this study were considerate to investigate the binding site of other two molecules, dual antagonists of chemokine receptor CCR2 and CCR5: TAK-779 and Teijin compound 1. Comparing the chemical structure of Teijin (Fig. 11B) and V-10-5191 is possible to notice some common structural features. In Fig. 11A residues His121$^{3.33}$ and Ile263$^{6.55}$ show favourable van der Waals interaction with the 2,4-dimethyphenil moiety of Teijin compound 1. Thr292$^{7.40}$ forms a

hydrogen bond to the carbonyl attached to the trifluorotoluyl ring and Glu291$^{7.39}$ shows electrostatic interaction with the pyrrolidine nitrogen. In contrast from what predicted by the homology model from Hall *et al.* Y120A$^{3.32}$ showed no significant interaction for Teijin compound 1. In our study Tyr120$^{3.32}$ seems to have a stronger effect on V-10-5191. According to our homology model this could be due to the ethoxy group on the pyrrolidine ring that can interact with Y120A$^{3.32}$ and represents one of the structural differences between the two molecules.

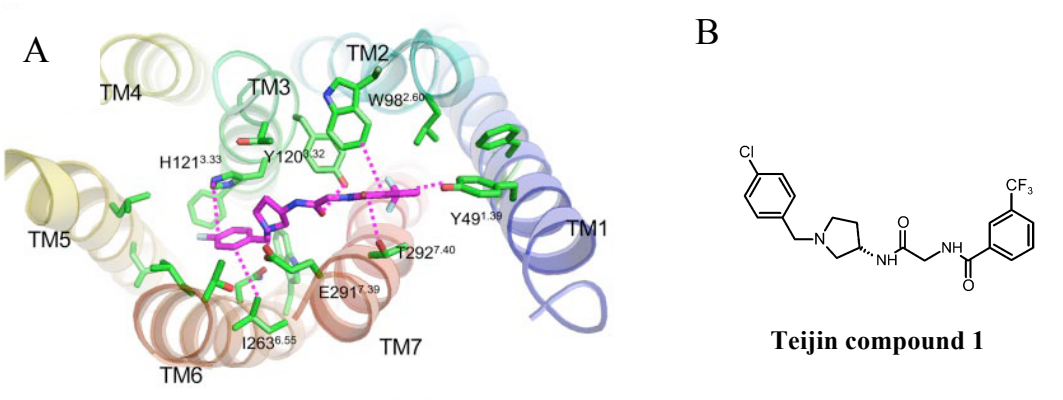

Fig. 11 Residues in binding pocket of Teijin compound 1 (A) and chemical structure of Teijin compound 1 (B) [52].

Chapter 5

Conclusion and future prospects

In conclusion, several techniques of transfection on HEK-293 cells have been applied in previous works [61] [62] but in the present study two different techniques of transfection on HEK-293 cells have been examined, showing that PEI transfection is the most efficiently. Concerning the use of PEI it could be interesting investigate the range of PEI toxicity in this cell line and distinguish which characteristics of plasmid constructs could influence receptors expression. Moreover, using site-directed mutagenesis and radioligand binding assay it has been possible to evaluate receptor expression and analyse the importance of some residues involved in the binding of CCR2 antagonists. In particular for the molecule V-10-5191 has been discovered that the conserved residues Tyr49$^{1.39}$, Trp98A$^{2.60}$, Thr292$^{7.40}$, but also Y120A$^{3.32}$ and Glu291$^{7.39}$ are significant for the binding on the receptor. According to literature, also some residue has been found to be crucial for CCL2 binding. These data allow identifying in TM1, TM2, TM3 and TM7 the binding pocket of V-10-5191 also recognized as “minor binding pocket” for other small molecules antagonists of chemokine receptor [34]. Using binding assay it would also be advisable evaluate the K_i for the mutants that affect mainly antagonists binding and through functional assay (e.g. GTPγS assay) determine their EC_{50} (half maximal effective

concentration). In order to better investigate the binding pocket of antagonists of chemokine receptor, several other mutants have been identified. One approach is to study other chemokine receptors and find out some mutants important in the binding. After the alignment of the sequence coding for WT human CCR2 and other chemokine receptor (appendix) some residues Asp78$^{2.40}$, Ser101$^{2.63}$, Arg206$^{5.42}$, Tyr305$^{7.53}$, Lys311$^{8.49}$, Lys180 and Lys183 have already been identified to be important for antagonists binding. They are localized in TM2, TM5, TM7, helix 8 and in the second extracellular loop, respectively. In particular Lys311$^{8.49}$ seems to be very promising to investigate the binding of V-10-5299 suggesting an intracellular binding site for the molecule. Finally, looking into the effect of these mutations on ligand affinity to the receptor, It could be possible propose the synthesis of new molecules antagonists of chemokine receptor having stronger interactions with the receptor. This might be translated with a better affinity and efficacy, or a longer residence time that hopefully will lead to a therapeutic improvement.

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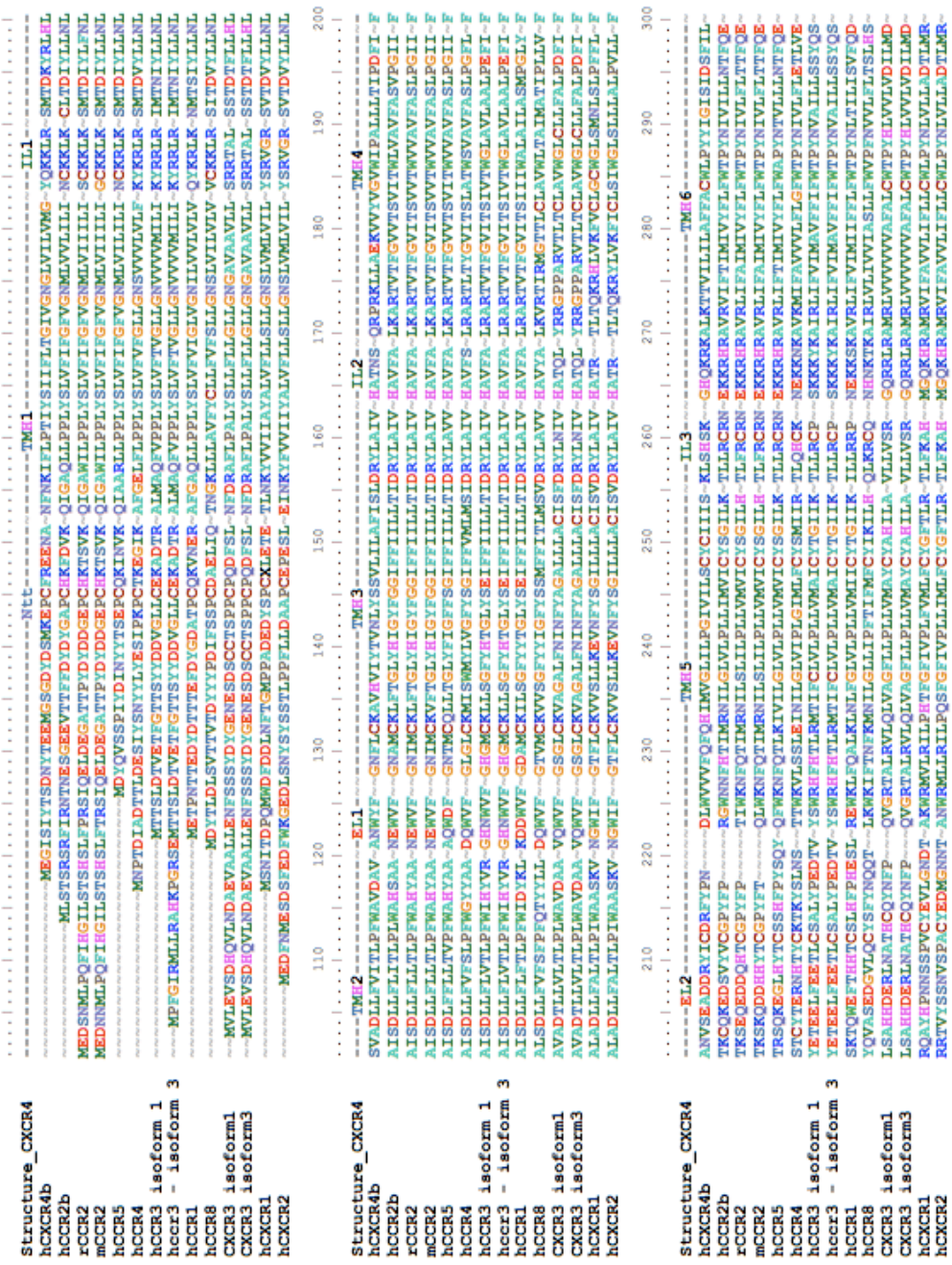
Appendix

Alignment of human, mouse and rat of CCR2 and other chemokine receptors



Structure_CXCR4
hCXCR4b
hCCR2b
rCCR2
mCCR2
hCCR5
hCCR4
hCCR3 isoform 1
hccr3 - isoform 3
hCCR1
hCCR8
CXCR3 isoform1
CXCR3 isoform3
hCXCR1
hCXCR2

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