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Studies in the activation mechanisms of chemokine receptors in the presence of Zn^{2+} coordination compounds and small molecule ligands with potential antiviral aplications

Ph.D. Thesis Summary

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Contents

Keywords: drug discovery, G protein-coupled receptor, molecular modeling, virtual screening, allostery, viral-encoded chemokine receptor, antiviral, therapeutic, inverse agonism, ago-allosteric modulator, positive allosteric modulation, Zn^{2+} chelate complex, 2,2'-bipyridine, 2,2':6',2"-terpyridine, 4'-chloro-2,2':6',2"-terpyridine, chemokine receptor activation mechanism, combined *in silico* and *in vitro* approach

The thesis entitled "**Studies in the activation mechanisms of chemokine receptors in the presence of Zn^{2+} coordination compounds and small molecule ligands with potential antiviral applications**" spans over **129** pages and is accompanied by **302** bibliographical references and **5** annexes. The experimental results obtained throughout the elaboration of this work are the subject of **11** tables, **45** figures and **2** publications in journals with impact factor, a book chapter in a series indexed in Thomson Reuters Web of Knowledge, as well as another manuscript in preparation. The summary represents a short review of the main results from the thesis, followed by general conclusions and selected references.

Introduction and objectives

Unraveling ligand-receptor interactions at membrane-spanning proteins involved in organism homeostasis has captivated the attention of the scientific community even before the emergence of the first structural insights into their spatial organization at the cell membrane [1, 2]. G protein-coupled receptors (GPCRs) are a superfamily of such transmembrane proteins, and play an indispensable role in the normal processes of the body through mediating signal transduction cascades. Notably, several sensory receptors, including visual, olfactory, taste and pain receptors are GPCRs, as are a plethora of receptors involved in neurotransmission and cognitive functions, as well as regulation of basic cell growth, differentiation and apoptosis [3-6]. At the same time, they are involved in numerous disease physiopathologies including cancer, depression and viral infections, and are one of the main targets of currently marketed drugs [7], as well as numerous drug discovery efforts [8]. Despite the diversity of ligands capable of inducing signals through their cognate receptors, the activation mechanisms of GPCRs are similar [9], but are not dictated by universal receptor-ligand interactions [10] and consequently, the drug discovery process is far from being straightforward [11].

Metal ions, as well as metal complexes, have demonstrated their utility in the structural analysis of GPCRs, as the use of engineered metal sites in establishing intra-receptor interactions has been described numerous times for various GPCR family members [12-17]. The interactions between metal ions and amino acid side chains are fairly specific, and occur in well-defined conditions, dependent on the preferred coordination geometry of the metal center. Moreover, if the scaffold of the used chelating agents contains aromatic moieties, the accommodation of the metal complexes at the receptor binding site will determine an extended conformational change of adjacent aromatic amino acid side chains through π-π or other aromatic interaction types, and these will serve as the initial substrate for receptor activation. The introduction of metal binding sites at GPCRs, along with the use of specific polypyridine chelating agents for stabilizing metal centers at the receptor binding site have laid the groundwork for understanding basic concepts of receptor activation [12, 13, 18, 19]. Several simple polypyridines can modulate the function of bound endogenous ligands in an allosteric fashion in the presence of metal ions [20-22], suggesting that coordination compounds can also be of great use in the study of allostery at GPCRs, and subsequently aid in the development of new allosteric modulators with distinct signaling properties for therapeutic purposes.

A particular class of GPCRs in the focus of many drug discovery efforts due to its involvement in inflammation, tumor development and viral infection, is that of the chemokine receptors [23]. The endogenous ligands for this type of receptors are 8-12 kDa chemotactic proteins, also known as chemokines, which interact with their cognate receptors through extensive amino acid contact networks, both in the extracellular and transmembrane region of the receptor, as opposed to other GPCRs of the same family, which usually bind endogenous small molecules in well-defined areas at the transmembrane region. Thus, studying the activation mechanisms of chemokine receptors in the presence of endogenous chemokine agonists can be cumbersome, and due to the large interaction area between chemokine:receptor pairs, alternative methods are required in order to understand the molecular mechanisms of receptor activation.

Many viral pathogens such as HIV use chemokine receptors to gain cell entry through the accommodation of a viral glycoprotein, which mimics the endogenous chemokines, at the receptor binding site. The human cytomegalovirus (HCMV) encodes proteins closely related to chemokines or chemokine receptors in order to evade immune surveillance, and establishes latent infections in most humans, which at healthy individuals is rarely symptomatic. However, HCMV primary infection is the major cause of mortality and morbidity in immunodefficient individuals such as organ transplant recipients, newborn infants, patients suffering from autoimmune disease, as well as AIDS and cancer patients. About one in 150 infants are born with congenital HCMV infection, which can cause serious health problems including mental retardation, visual or hearing impairment, microcephaly, seizures or encephalitis in 10-15% of cases, or can induce pregnancy loss or premature birth, making HCMV infection one of the major worldwide causes of birth defects [24, 25]. Needless to say, the demand for HCMV-targeted therapies is great. Since the viral-encoded chemokine receptor US28 is expressed in all stages of viral infection including latency, it may be a suitable target for HCMV-directed therapies, but unfortunately, the number of available molecular scaffolds able to act at this receptor is limited.

Ongoing efforts to develop drugs acting at the chemokine system have led to the approval of only two small-molecules drugs on the pharmaceutical market: Maraviroc (Celsentri$^{©}$), a negative allosteric modulator for CCR5 used for inhibiting HIV-entry at R5-tropic strains, and plerixafor (Mozobil$^{©}$), used for mobilizing bone marrow hematopoietic stem cells in patients undergoing chemotherapy. Other potential anti-inflammatory or antiviral candidates did not pass clinical trials [26]. Consequently, a better understanding of the chemokine system, combined with continuous drug development efforts could lead to an increase in the number of successful marketed drugs.

Thus, the research undertaken in the thesis focuses on understanding the molecular mechanisms behind small molecule-induced chemokine receptor activation and allosteric modulation, as well as the identification of novel small molecule ligands or molecular scaffolds which target the chemokine system, with the end purpose of generating new therapeutic strategies which are aimed at the chemokine system, such as HCMV infection.

The general objectives of the thesis were as follows:

1. To describe the molecular mechanisms behind chemokine receptor activation and allosteric modulation by small molecule ligands with a metal center through a combined *in silico* and *in vitro* approach;
2. To identify novel molecular scaffolds able to modulate chemokine receptor activity or influence chemokine binding to receptors though a combined *in silico* and *in vitro* approach;

The abovementioned specific objectives were accomplished through the completion of the following specific objectives:

1. Propose a binding site for the metal-ion chelator complexes ZnBip, ZnTerp and ZnClTerp at the chemokine receptor CCR5 though *in silico* docking experiments;
2. Validate the proposed binding sites through *in vitro* experiments at the wild-type CCR5 receptor (WT) and 23 receptor mutants, generated based on computational results;
3. Identify the amino acids involved in the accommodation of all three metal-ion chelators in the binding site of CCR5.
4. Describe the structural basis of ago-allosteric activity for ZnBip and ZnTerp and pure allosteric activity for ZnClTerp at the chemokine receptor CCR5 based on *in silico* and *in vitro* results;

5. Create a library of small molecule ligands which possess the same pharmacophore features as the known US28 inverse agonist, VUF2274, extracted from databases of commercially available compounds;
6. Evaluate the library of small molecule ligands in functional and competition binding assays at US28;
7. Identify the novel scaffolds capable of modulating US28 activity and map their binding sites at the viral-encoded chemokine receptor;
8. Create a library of small molecule ligands which contain the same pharmacophore features as the first generation lead structures **33**, **125** and **76**, and evaluate them through both functional and competition binding assays at US28;
9. Identify novel ligands capable of modulating US28 activity with improved pharmacological properties than parent compounds and map their binding sites at the viral-encoded chemokine receptor;
10. Demonstrate the utility of the truncated receptor construct US28Δ300 in the drug discovery process at the US28 receptor.

In vitro experiments, as well as some of the *in silico* experiments presented in the thesis were performed at the Institute of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark, in the research groups of Mette M Rosenkilde and Thomas M Frimurer, during three separate mobility periods undertaken during my doctoral studies. In the thesis summarized herein, the used methodology, both computational and *in vitro* is detailed in the section *Materials and Methods*.

The *Original Research* section of the thesis is divided into three chapters, each focusing on a relevant subject which ultimately contributes to the understanding the chemokine system and aids in the development of new receptor-targeted therapies. In the following pages, the relevant experimental results of each chapter are presented. The numbering of chapters, figures, tables and structures in this summary is identical to the numbering used in the thesis.

Chapter 3. Structural insights into the allosteric modulation of chemokine receptor CCR5 by simple polypyridine chelators

The first four objectives were addressed to in the chapter 3 of the thesis. The three chelators considered in this study were 2,2'-bipyridine, 2,2':6',2"-terpyridine and 4'-chloro-2,2':6',2"-terpyridine (Figure 15).

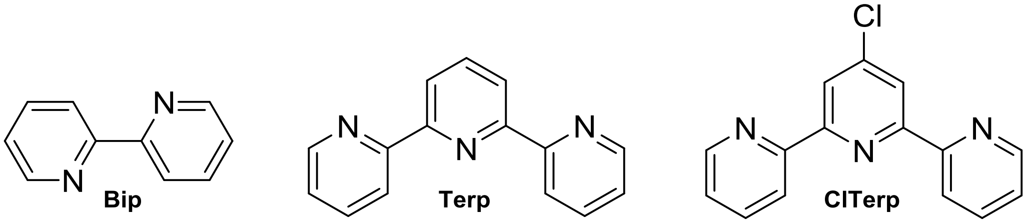

Figure 15. Chemical structures of 2,2'-bipyridine (Bip), 2,2':6',2"-terpyridine (Terp) and 4'-chloro-2,2':6',2"-terpyridine (ClTerp)

Despite an apparent minor structural difference, Terp and ClTerp exhibit differential pharmacological behaviors at CCR5 in the presence of aqueous Zn^{2+}, with the ZnTerp complex being an ago-allosteric modulator which can both enhance CCL3 binding and independently activate the receptor (similar to ZnBip), while the ZnClTerp complex behaves as a pure allosteric enhancer and can only influence CCL3 binding to the receptor, and is not able to independently induce activation [22, 27].

Thus, in the case of the two terpyridines, the allosteric effect can be separated from agonistic activity though the simple introduction of a chloro substituent, but it is not clear if this behavior arises due to the two compounds being accommodated in a distinct fashion at the receptor or if the compounds establish preferential contacts with amino acids lining the same receptor binding pocket. Aiming to understand the structural basis of allostery and ago-allostery for the two terpyridines and bipyridine at CCR5, as well as the molecular mechanisms behind the separation of effect, the binding sites of the metal-ion chelator complexes were first predicted using computational modeling, and then confirmed though *in vitro* functional and binding experiments at 23 receptor mutants.

First, a model of CCR5 was generated, based on the structural coordinates of the viral-encoded chemokine receptor US28, which bears the hallmarks of the active state, since all the tested ligands are either direct activators of the receptor or enhance the binding of chemokines to the receptor. Next, since the metal complexes are formed *in* situ, a Zn^{2+} ion was placed in the vicinity of the proposed metal anchor in TM VII, E283.

After docking experiments were performed, it was revealed that all three metal-ion complexes are accommodated in a similar fashion to the receptor. At the same time, all chelators exert their activity at CCR5 through the formation of a coordination complex with the zinc ion and the side chain of the highly conserved glutamic acid in TM VII, E283. All compounds largely overlap in binding sites and are further stabilized at the receptor through hydrophobic interactions with aromatic amino acids lining both the minor and major binding subpockets, such as Y37, W86, Y108, F109, F112, W248 and Y251, as well as R168 from ECL2 (Figure 17).

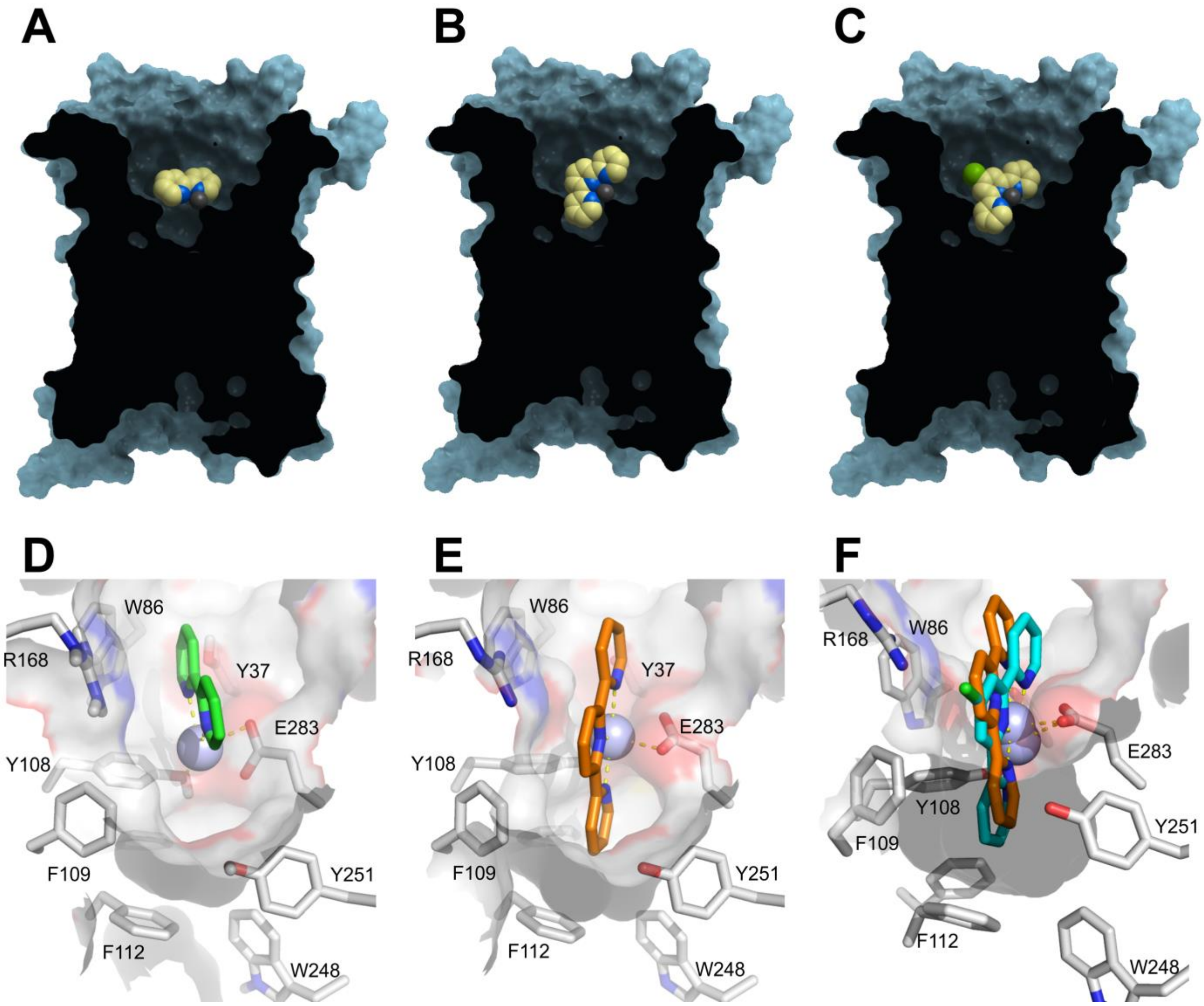

Figure 17. Binding modes of (A,D) ZnBip, (B,E) ZnTerp and (C) ZnClTerp at CCR5; (F) comparison between the binding modes of ZnTerp (orange) and ZnClTerp (cyan) at CCR5, extracellular view

The third pyridine ring in the two terpyrdine complexes in accommodated deep in the major binding crevice. In addition, the chloro substituent of ClTerp points towards the extracellular surface and interacts with F109, possibly through a Cl-π interaction, but fails to establish a favorable contact with W248, also known as the toggle switch tryptophan, which is generally considered crucial for ligand-mediated receptor activation. Thus, the lack of intrinsic activity observed at ZnClTerp could be at least in part caused by the unfavorable interaction of this complex with the toggle switch tryptophan or the stabilization of its side chain in a position which prevents receptor activation.

Based on the docking results, a series of 23 receptor mutants was generated (Figure 20), both in the minor and major subpockets, in which amino acids thought to interact with the metal-ion chelator complexes were mutated to alanine or aromatic/hydrophobic amino acids. Compounds were then evaluated through functional assays in order to asses the impact of the mutation of the compounds' intrinsic activity, as well as through competition biding experiments in order to assess the impact of the mutation on the compounds' allosteric effect.

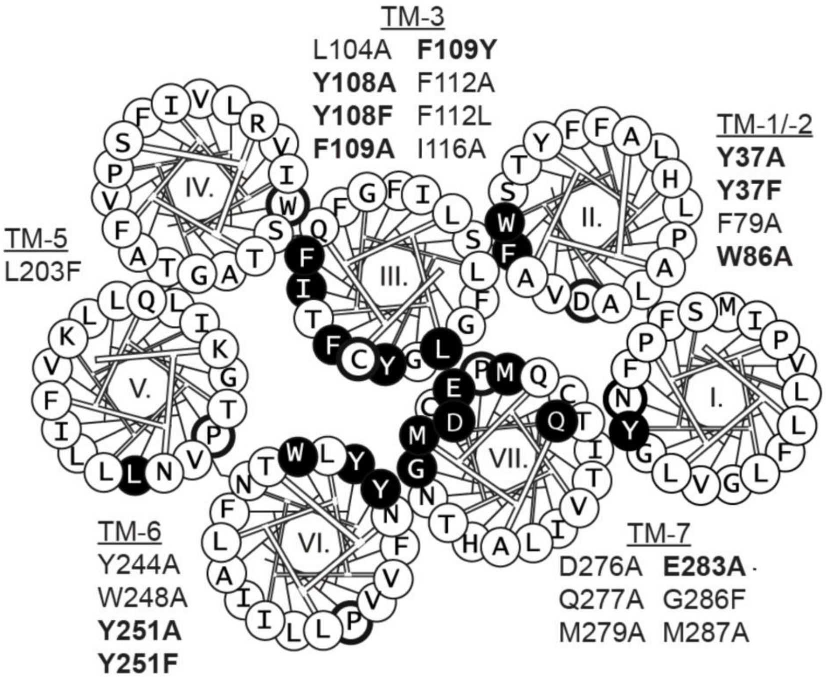

Figure 20. Helical wheel diagram of CCR5 showing the residues in the EC halves of the TM helices. The mutated residues are represented as filled black circles, and the most conserved residue in each TM helix is represented as a thick black-bordered circle; all mutations included in this thesis are listed next to their corresponding helices; relevant mutants are given in bold: major binding pocket: Y108A/F, F109A/Y, Y251A/F; minor binding pocket: Y37A/F, W86A; metal anchor: E283A.

Functional evaluation of the complexes, as well as the endogenous chemokines CCL3 and CCL5 at the mutant receptors (Figure 21), combined with evaluation of the allosteric activity through competition binding experiments against radiolabeled chemokines (Table 3) strongly supported the previously presented *in silico* results.

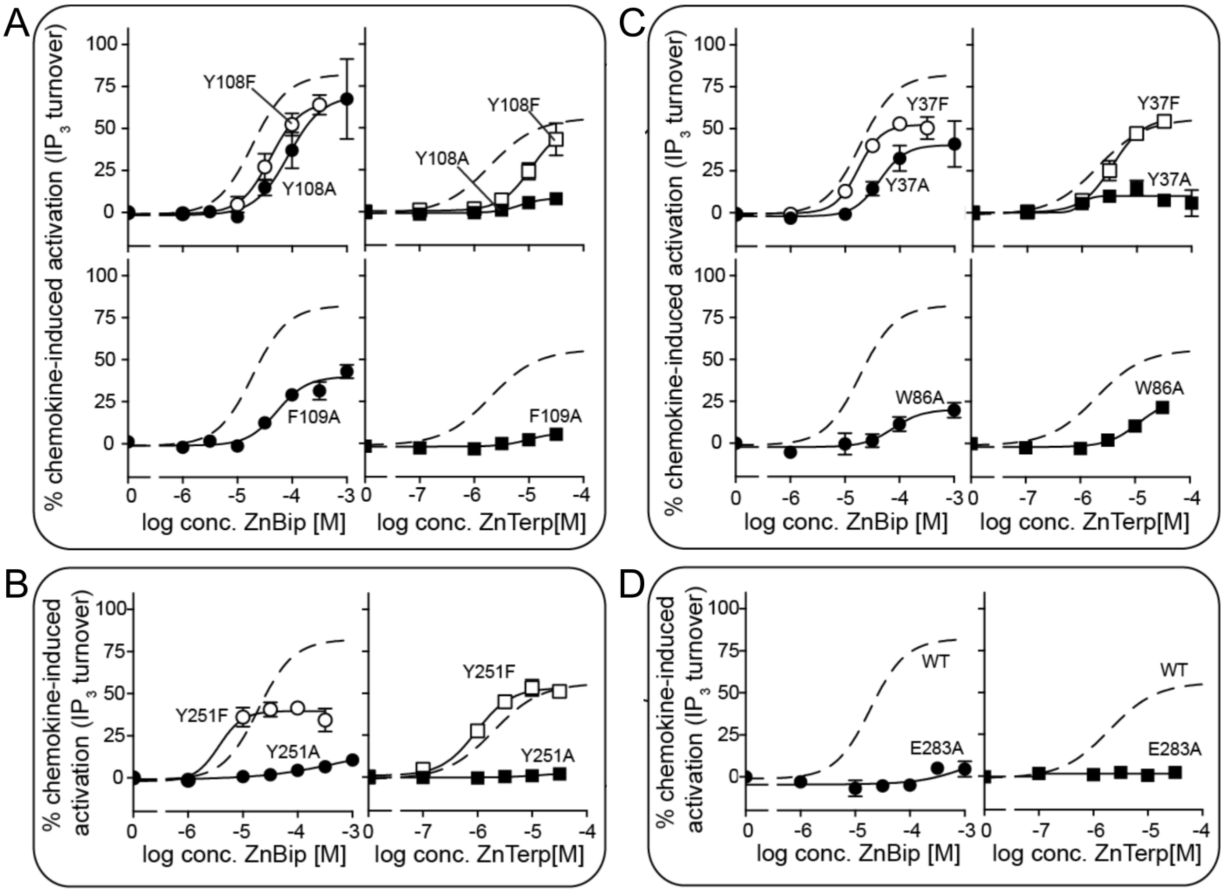

Figure 21. Activity of ZnBip (left lanes, circles) and ZnTerp (right lanes, squares) at selected receptor mutants as measured by IP accumulation assays; metal-ion chelator complex activity at WT is represented as a dashed line in each graph; (A) activity at relevant mutants in TM III of the major binding pocket – Y108A/F and F109A; (B) activity at Y251A/F in TM VI of the major binding pocket; (C) activity at relevant mutants in TM I/II of the minor binding pocket – Y37A/F and W86A; (D) activity at E283A mutant in TM VII.

Mutations such as W86A, F109A, W248A, Y251A or E283A had a tremendous impact on the intrinsic activity of ZnBip and ZnTerp, while F79A and Y244A were found important to the overall integrity of the receptor, as none of the tested chemokines could induce activation at these receptor mutants.

In terms of binding, W86A selectively impacted the ability of ZnBip to enhance the binding of CCL3 to the receptor, while Y37A selectively impacted the same ability in the case of the two terpyridine complexes. As expected, E283A and F109A were crucial for the allosteric activity of all metal ion-chelator complexes, while only ZnTerp was impacted by the W248A mutation, further supporting the initial *in silico* hypothesis.

Table 3. Heterologous radioactive competition binding assays with ^{125}I-CCL3 as radioligand and ZnBip, ZnTerp or ZnClTerp as competitors; the name and position of each mutated residue is given according to the Ballesteros-Weinstein and Schwartz systems; K_i values are given in log and μM; F_{mut} represents the K_i fold decrease for the mutant compared to WT CCR5; color code: > 2 yellow; >5 orange; >10 red; ZnClTerp displaces ^{125}I-CCL3 at Y37A, highlighted in blue.

			ZnBip				**ZnTerp**				**ZnClTerp**			
		BW/S	Affinity				Affinity				Affinity			
			$\log K_i$ ± SEM	(μM)	F_{mut}	(n)	$\log K_i$ ± SEM	(μM)	F_{mut}	(n)	$\log K_i$ ± SEM	(μM)	F_{mut}	(n)
	WT		-4.2 ± 0.13	66	**1.0**	(8)	-4.8 ± 0.09	16	**1.0**	(7)	-5.2 ± 0.07	6	**1.0**	(4)
TM I	**Y37A**	I:07/1.39	-4.2 ± 0.01	67	**1.0**	(3)	-4.4 ± 0.29	37	**2.4**	(3)	-5.7 ± 0.05	2.1	**0.34**	(3)
	Y37F	I:07/1.39	-4.0 ± 0.02	95	**1.4**	(3)	-4.7 ± 0.07	21	**1.3**	(3)	-5.0 ± 0.19	11	**1.8**	(3)
TM II	**W86A**	II:20/2.60	-3.4 ± 0.16	420	**6.3**	(4)	-4.6 ± 0.10	24	**1.5**	(3)	-5.0 ± 0.02	9.7	**1.6**	(3)
TM III	**Y108A**	III:08/3.32	-4.1 ± 0.01	79	**1.2**	(3)	-4.8 ± 0.16	18	**1.1**	(4)	-5.0 ± 0.10	11	**1.8**	(3)
	F109A	III:09/3.33	no enhanced binding		🟥	(3)	no enhanced binding		🟥	(3)	no enhanced binding		🟥	(3)
	F112A	III:12/3.36	-4.0 ± 0.07	99	**1.5**	(4)	-4.6 ± 0.14	23	**1.4**	(5)	-5.2 ± 0.08	6.3	**1.0**	(4)
TM V	**L203F**	V:13/5.47	-4.0 ± 0.10	90	**1.4**	(3)	not determined				not determined			
TM VI	**W248A**	VI:13/4.48	-4.1 ± 0.05	85	**1.3**	(4)	no enhanced binding		🟥	(4)	-5.3 ± 0.16	5.5	**0.9**	(4)
	Y251A	VI:16/4.51	-4.1 ± 0.09	75	**1.1**	(4)	-4.8 ± 0.06	17	**1.1**	(4)	-5.1 ± 0.05	8.5	**1.4**	(4)
TM VII	**D276A**	VII:-02/7.32	-3.7 ± 0.21	204	**3.1**	(3)	-4.5 ± 0.11	28	**1.8**	(4)	-5.1 ± 0.1	8.6	**1.4**	(4)
	E283A	VII:06/7.39	no enhanced binding		🟥	(3)	no enhanced binding		🟥	(4)	-4.0 ± 0.07	105	**17**	(3)
	G286F	VII:09/7.42	-4.4 ± 0.00	36	**0.55**	(3)	not determined				not determined			
	M287A	VII:10/7.43	-3.7 ± 0.21	204	**3.1**	(3)	-5.1 ± 0.12	7.6	**0.48**	(3)	-5.2 ± 0.03	6.8	**1.1**	(3)

In conclusion, chapter 3 is focused on understanding the structural mechanisms behind receptor activation and allosteric modulation by the small metal-ion chelator complexes ZnBip, ZnTerp and ZnClTerp at CCR5 though a combined i*n silico* and *in vitro* approach. Based on molecular docking experiments, a series of 23 mutant receptors was generated, and both the metal-ion chelator complexes and endogenous chemokine ligands were tested at each receptor mutant through IP accumulation experiments and competition binding experiments using radiolabeled chemokines.

In silico docking experiments indicated that all three tested metal-ion complexes are accommodated to the receptor in a similar fashion, while *in vitro* assays, including mutagenesis experiments strongly supported the proposed model.

At all three complexes, the metal ion anchor is consists of the highly conserved glutamic acid in TM VII, E283. Other amino acids involved in the stabilization of the chelator scaffold in the CCR5 binding site include Y37, W86, Y108, F109, F112, W248 and Y251. Furthermore, it was revealed that the third pyridine ring in ZnTerp and ZnClTerp protrudes deep in the major binding crevice delimited by W248 and Y251, and that the lack of intrinsic activity observed for ZnClTerp could be explained by a faulty interaction of the chelator with W248, the toggle switch tryptophan, which is generally involved in ligand-mediated receptor activation.

Overall, the results presented in chapter 3 illustrate that small molecule ligands such as metal-ion chelator complexes can be specifically tailored to modify the behavior of specific chemokine:receptor pairs. With this respect, the simple introduction of a chloro substituent (Terp vs. ClTerp) can significantly alter the behavior of a ligand both in terms of intrinsic activity and allosteric effect. Other properties could be further explored for the future rational design of particular ligand types with medicinal applications, including modulators of chemokine function, receptor antagonists or ago-allosteric modulators.

Chapter 4. The identification and pharmacological characterization of novel ligands acting at the viral-encoded chemokine receptor US28

Although the use of metal ions or metal-ion chelator complexes has proven its utility in studying activation mechanisms and allosteric modulation at chemokine receptors [16, 21, 28] or GPCRs in general [13, 15, 29], small molecule ligands without a metal center possess a greater pharmacological potential in the chemokine system. With the exception of CXCR4-targeting azamacrocycles with antiviral potential [30-32], most currently investigated ligands targeting the chemokine system are small organic molecules [33]. The generation of SAR descriptors for small organic molecules is easy to perform, and the availability of millions of chemical structures in compound databases permits the identification of novel scaffolds with similar pharmacophore features to a lead drug, possibly with improved therapeutic effects.

A current pharmacological focus is the identification of novel small molecule ligands for anti-HCMV therapy by targeting a viral-encoded chemokine receptor involved in virus dissemination, as well as in viral-associated tumor formation and vascular disease, US28 [34]. This receptor is also present in latent-infected cells, making it a suitable target for HCMV-directed therapies regardless of disease progression and viremia [35]. US28 possesses a remarkable set of characteristics, from constitutive signaling through various pathways [36-39] to chemokine scavenging [40], most of which have been targeted in the development of various pharmaceutical strategies. Notably, the chemokine scavenging property of US28, combined with its ability to bind CX_3CL1 with high affinity, have been utilized in the successful development of fusion toxin proteins which specifically destroy HCMV-infected cells by binding to US28 [41, 42].

Dampening the observed constitutive activity levels of US28 is an attractive receptor property which can be explored in order to develop novel potential antiviral therapies. The first reported nonpeptidergic molecule to display activity on US28, VUF2274, acted as an inverse agonist in the PLC signaling pathway at the US28 receptor [43], but is also a very potent antagonist for the closely-related chemokine receptor CCR1 [44], a property which limits its use in the clinic due to possible cross-reactivity. Other compounds containing various scaffolds, including tetrahydroisoquinoline, flavonoid, chalcone and azepine, have been developed in order to lower the constitutive levels of US28, but their relatively low potencies limit their further therapeutic application.

The current set of available US28-targetting molecules has been identified through strategies such as hybrid compound generation, screening in-house compound databases or applying structural modifications to previously identified scaffolds, therefore the number of available ligands with unique scaffolds able to modulate US28 activity is limited. Thus, novel strategies such as screening entire compound databases should be adopted in order to unravel new US28 ligands with improved potencies in order to explore the constitutive activity of US28 in anti-HCMV therapies and to gain more insights into the physiological relevance of US28 in viral pathogenesis [45].

Therefore, the focus of chapter 4 was to identify novel chemical scaffolds capable of acting at the US28 receptor starting from the molecular descriptors of a known US28 ligand which is able to lower the constitutive activity levels of US28 (also known as inverse agonist), VUF2274.

First, a model of US28 was generated through homology modeling based on the structural coordinates of the closely related CCR5 receptor. A possible binding site for VUF2274 was then identified based on the superimposition of the US28 model on the template structure and the relative orientation of Maraviroc in the CCR5 crystal structure. VUF2274 was then docked to the receptor and its best-scoring conformation was refined and subsequently used as a seed structure for virtual screening.

The ligand-based virtual-screening workflow consisted of systematically searching through databases of commercially available compounds and selecting the most similar with VUF2274 in terms of molecular descriptors and spatial compatibility with the US28 binding site. Initially, over 12 million compounds from the ZINC database [46], which were stripped from counterions, assigned tautomers, protonation sates and correct charges were compared with VUF2274. For this purpose, the 2D pharmacophore features of each compound in the database were compared with the pharmacophore features of VUF2274 through a multiplet similarity search. The top 5% most similar structures ranked according to the Tanimoto coefficient (693,130 compounds) were then selected for a new step of database refinement, called Atomic Property Fields (APF) superimposition. This approach generates a grid for each compound based on seven 3D pharmacophore characteristics: (1) hydrogen bond donors (HBD), (2) hydrogen bond acceptors (HBA), (3) sp^2 hybridization, (4) lypophilicity, (5) size, (6) positive/negative charge and (7) electronegative/electropositive groups, which is then compared to the grid of the refined VUF2274 conformation extracted from the previously generated US28:VUF2274 model. Subsequent chemical filtering of the results using selection criteria aimed to assure bioavailability, such as molecular weight (< 500), number of rotatable bonds (nrotb < 12) or *n*-octanol/water partition coefficient (logP < 5.5), followed by compound clustering generated 1,454 cluster representatives, from which 294 were further selected based on sharing a set of specific pharmacophore features with VUF2274. After manual assessment of each of the 294 compounds in the US28 binding pocket, a total of 98 compounds were purchased for further *in vitro* pharmacological testing. A summary of the compound filtering and selection steps, as well as the number of structures generated after each step can be consulted in Figure 26.

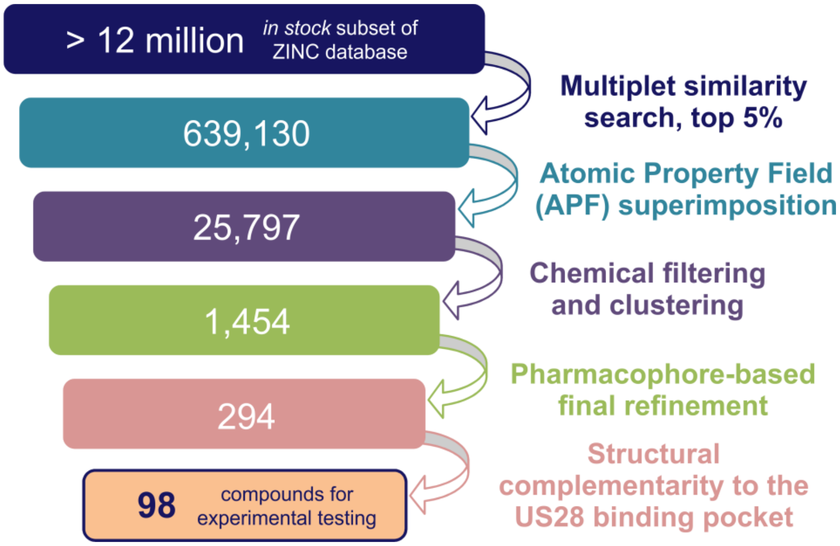

Figure 26. Compound filtering and selection steps, including the number of compound structures generated after each step.

In preliminary IP accumulation assays, most compounds showed a marked tendency towards inverse agonism, the most efficacious compound being **125**, which was able to lower about 44% of the basal US28 constitutive levels with an EC_{50} value of 1.76 µM, 2-fold improved compared to parent compound VUF2274. At the same time, compound **33** was identified as an agonist with moderate micromolar potency, having an EC_{50} value of 0.95 µM (Figure 27). Other assays such as calcium accumulation experiments confirmed their activity further downstream the G protein signaling cascade.

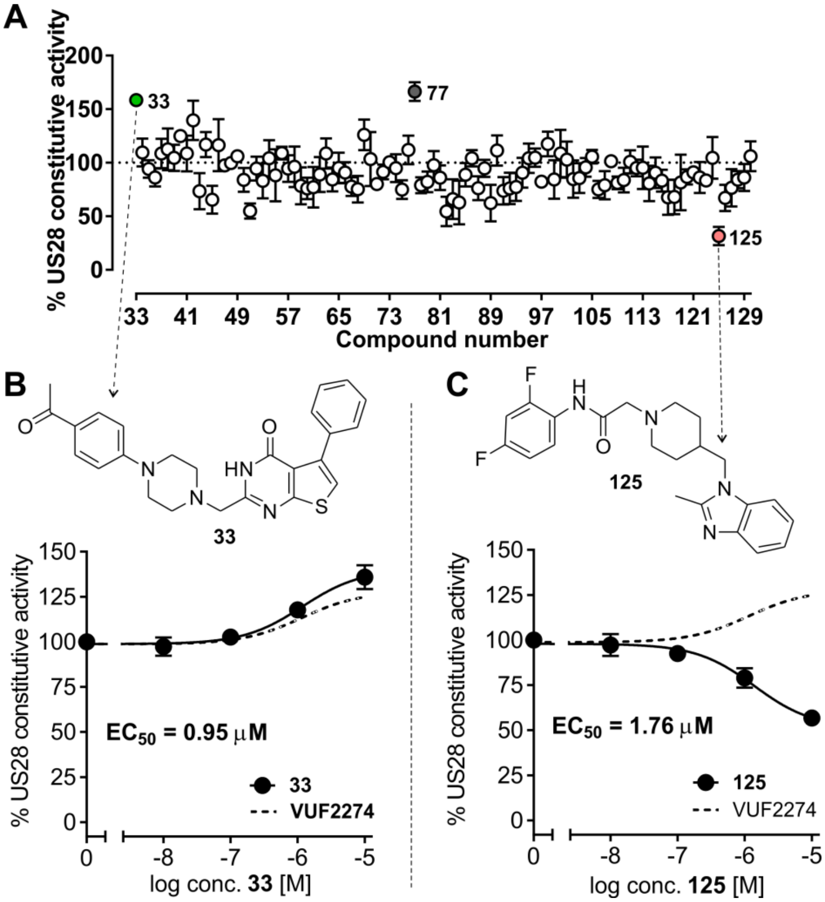

Figure 27. (A) Scatterplot of the initial single dose screening (10 μM) of the 98-compound library in IP accumulation experiments at US28 WT; the agonist (**33**) and inverse agonist (**125**) are indicated as green and orange dots, respectively; the nonspecific agonist **77** is colored in gray; (B,C) chemical structures and dose-response curves of **33** and **125**; the dose-response curve of VUF2274 is represented as a dotted black line in each graph; data are normalized between the basal activity of non-transfected cells (0%) and the basal constitutive levels of US28-expressing cells in the presence of 1% DMSO (100%) and represent the mean of at least 3 independent experiments performed in duplicate ± SEM;

It was furthermore found that both compounds exert their activity in an allosteric fashion in relation to CX_3CL1, as none could compete with the radiolabeled chemokine for US28 binding. In fact, only one out of 98 compounds was able to partially compete with this chemokine, namely compound **76**. However, LC-MS analysis showed its defragmentation even under mild ionization conditions, indicating that this compound might also be unstable in the *in vitro* experimental conditions.

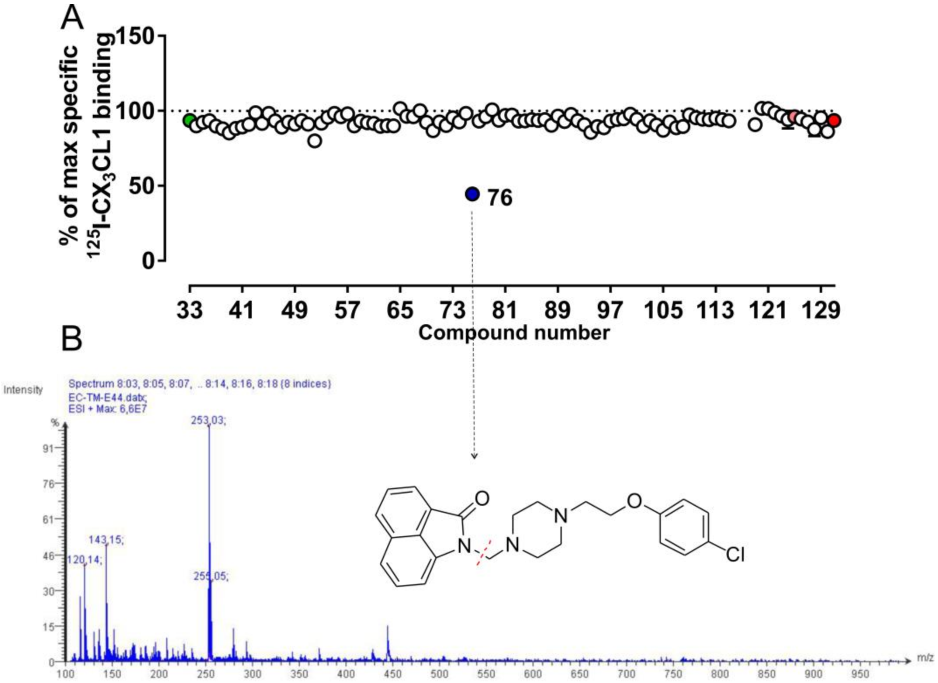

Figure 29. (A) Scatterplot of the initial single dose screening (10 µM) of the 98-compound library in heterologous competition binding experiments against ^{125}I-CX$_3$CL1; compound **76** is represented as a blue dot; **33**, **125** and **VUF2274** are colored in green, orange and red, respectively; data are normalized between the measured radioactivity in the presence of 1 µM unlabeled CX$_3$CL1 (0%) and 1% DMSO (100%); n=3; (B) ESI-MS spectra of compound **76** along with its structure and possible fragmentation site; major m/z peaks: 120.14; 143.15; 253.03; 255.05.

The structures of compounds **33** and **125** share a similar topology, with the right-hand side of the molecule consisting of a bicyclic system linked by a methylene bridge to a central heterocyclic system, piperazine in the case of **33** and piperidine for **125.** Both compounds are accommodated in the US28 binding pocket in a similar fashion, with the central positively charged nitrogen atom of both compounds forming a salt bridge with the chemokine receptor-family conserved glutamic acid in TM VII, E277 (Figure 31). The bulky substituents in the right-hand side of the molecules further engage in favorable aromatic interactions with W89 in TM II, Y112 in TM III and Y244 in TM VI. Slight differences were observed in each of the compounds' binding sites, such as in the relative orientation of W89 or the additional possible hydrogen bond interaction between the carbonyl oxygen of **125** and the hydroxyl group of Y112. Future mutagenesis studies could confirm the implication of these residues in the stabilization of the ligands in the US28 binding site.

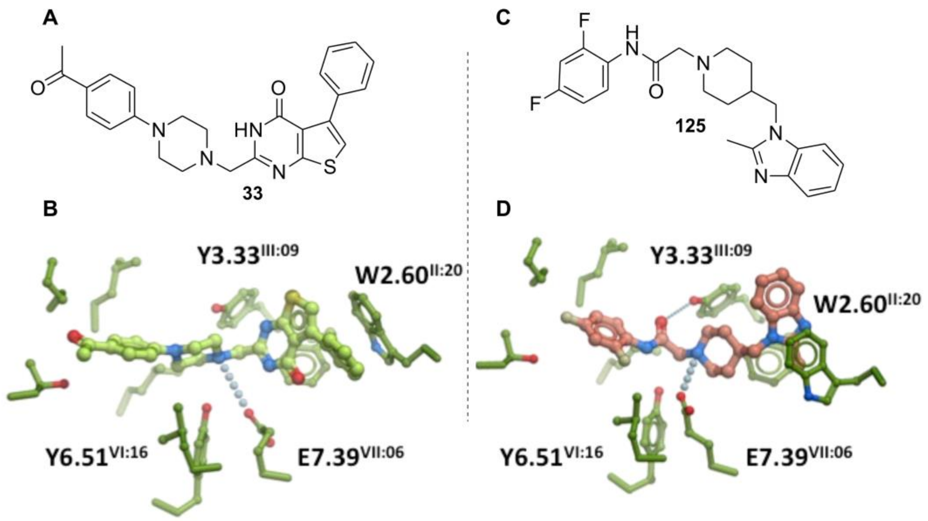

Figure 31. Chemical structures and binding conformations of (A,B) **33** and (C,D) **125** at US28; polar receptor-ligand interactions are represented as dashed blue lines; selected residue lining the binding site are labeled according to the Ballesteros/Schwartz numbering systems.

In summary, the main focus of chapter 4 was the identification and characterization of novel chemical scaffolds capable of acting at US28. Thus, starting from the molecular descriptors of a known US28 ligand, the ZINC database containing >12 million commercially available compounds was screened, and results were ranked, filtered and clustered for the generation of a final library of 98 compound with potential US28 activity.

In vitro assaying revealed a novel inverse agonist, **125**, capable of lowering 44% of basal US28 constitutive activity with an EC_{50} value of 1.76 μM, as well as a novel agonist, **33**, capable of potentiating basal US28 activity by 36% with an EC_{50} value of 0.95 μM.

The two novel ligands exert their activity in an allosteric fashion in relation to the chemokine CX_3CL1, and only one compound out of all tested ligands is able to partially compete with this chemokine, but its stability in experimental conditions is questionable.

Finally, **33** and **125** are both accommodated in a similar fashion at US28, as they share a similar topology consisting of a central heterocyclic moiety, flanked by bulky aromatic substituents both in the left- and right-hand sides of the molecules. The amino acids involved in the stabilization of these compounds in the US28 binding site include E277, which serves as an anchor for the positively charged heterocyclic nitrogen, as well as W89, Y112 and Y244.

The newly identified scaffolds represent valuable starting points for the generation of novel anti-HCMV therapies targeting the viral-encoded chemokine receptor US28.

Chapter 5. The pharmacological characterization of US28-acting ligands generated based on the scaffolds of previously identified lead structures

The final objectives of this thesis were addressed to in chapter 5. Thus, based on the structures of hits identified in the previous chapter, **33**, **125** and **76**, the same computational methodology was applied in order to generate a second generation library of 93 potential US28-acting ligands which were then evaluated *in vitro.*

After the initial single point evaluation in functional assays, compounds with measurable activity at US28 WT were further assayed for the calculation of EC_{50} values (Figure 37).

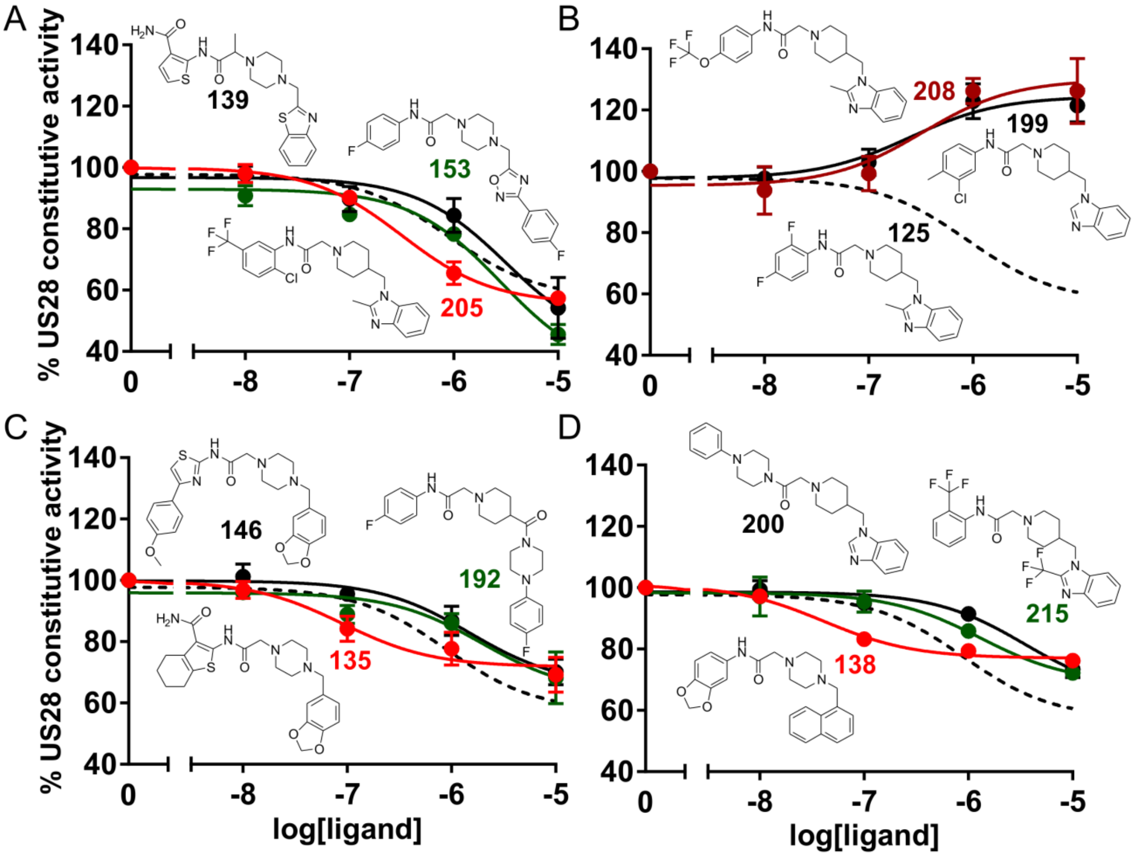

Figure 37. **S**tructure and function of novel ligands at the US28 receptor, as measured by IP accumulation experiments: (A) Activity of compounds capable of lowering US28 basal activity by more than 40%; (B) Activity of identified agonists at the US28 receptor; (C,D) Activity of compounds capable of lowering basal activity by more than 30% and 20%, respectively; data are normalized between the basal activity of non-induced cells (0%) and the basal constitutive levels of tetracyclin-induced US28-expressing cells in the presence of 1% DMSO (100%) and represent the mean of at least 3 independent experiments performed in duplicate ± SEM;

Thus, out of the compounds capable of lowering US28 basal activity levels by more than 40% in highly constitutive active systems, compound **205** was slightly more potent than **125** (2.8-fold), while **153** and **139** were 3-fold or 4-fold less potent, respectively (Figure 37A). Compound **205** is the most similar in terms of structure with **125** from all identified inverse agonists, suggesting that it retains some of its parent's molecular activity determinants. The two compounds capable of potentiating basal US28 activity **199** and **208** (Figure 37B) also bear most of the structural hallmarks of **125**, making the interpretation of eventual structure-activity relationships more difficult, as has been previously observed at other US28 ligands.

When high basal constitutive activity levels were exhibited by the system, compound **135** showed the most optimal efficacy/potency combination, having slightly lower efficacy than **125**, but 11-fold improved potency (Figure 37C). Compound **146**, which bears the same right-hand side substituent as **135**, but has a bulkier substituent with a sequestered nitrogen atom in a thiazole ring in the left-hand side is 20-fold less potent than **135**, suggesting that both the volume of the substituent, the net charge and spatial availability of the nitrogen atom are important for observed activity at the US28 receptor.

Finally, compound **138** was able to lower 23% of the basal US28 constitutive activity with nanomolar potency (EC_{50} ~ 38 nM), but this behavior could only be observed at high levels of constitutive activity (Figure 37D), this compound displaying no observable activity in preliminary single dose experiments.

None of the compounds could completely displace CX_3CL1 binding, although some compounds were able to weakly compete with it (e.g. **134** and **208**, 32% chemokine displacement at 10 μM). As expected, VUF2274 did not compete with ^{125}I-CX_3CL1, nor did **125**, as previously noted (Figure 38A).

Five out of the 93 compounds were able to displace radiolabeled CCL2 by more than 40%, compound **208** displaying the best efficacy (78% displacement). An additional six compounds could compete with more than 15% of receptor-bound ^{125}I-CCL2. VUF2274 displaced about 60% ^{125}I-CCL2, while **125** could only compete with about 10% of the radiolabeled chemokine, as previously observed (Figure 38B).

Intrigued by these findings, and in order to ascertain if any chemokine-dependent behaviors exist, the ability of the small molecule compounds to displace CCL4 was also assessed, and it was found that most of the compounds identified as CCL2 displacers were able to compete with CCL4 as well, with comparable efficacies (Figure 38C).

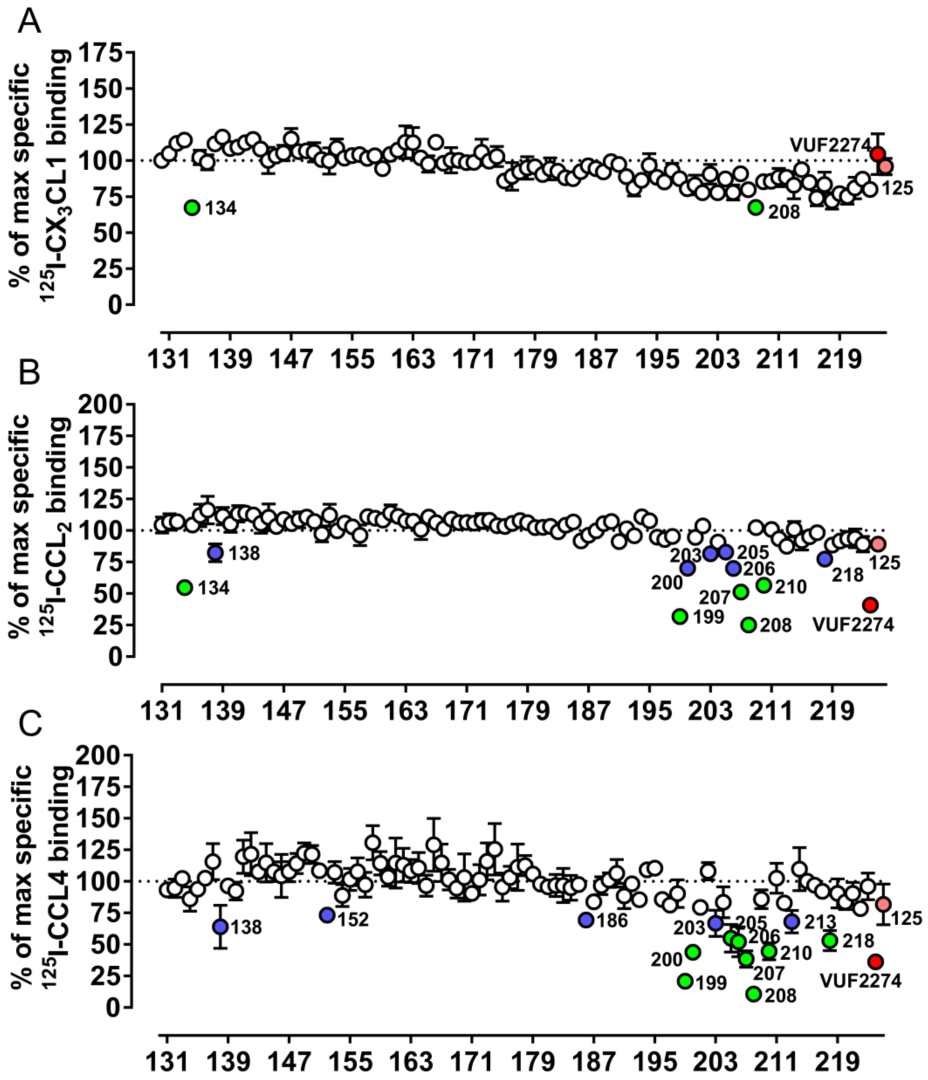

Figure 38. Scatterplot of the initial single dose screening (10 μM) of the 93-compound library in heterologous competition binding experiments against (A) ^{125}I-CX$_3$CL1; (B) ^{125}I-CCL2; (C) ^{125}I-CCL4; green dots: >40% chemokine displacement; blue dots: >20% chemokine displacement; **125** and **VUF2274** are colored in orange and red, respectively; data are normalized between the measured radioactivity in the presence of 1 μM unlabeled chemokine (0%) and 1% DMSO (100%); n=3.

In fact, except for a few compounds which seem to have differential chemokine displacement efficacies (*e.g.* **134** selectively competes with ^{125}I-CCL2), the degree to which the compounds compete with the two chemokines correlate well in a linear fashion (Figure

39), suggesting that these compounds have a common binding mode which is capable of recognizing both CC chemokine-stabilized receptor conformations, but not CX_3CL1.

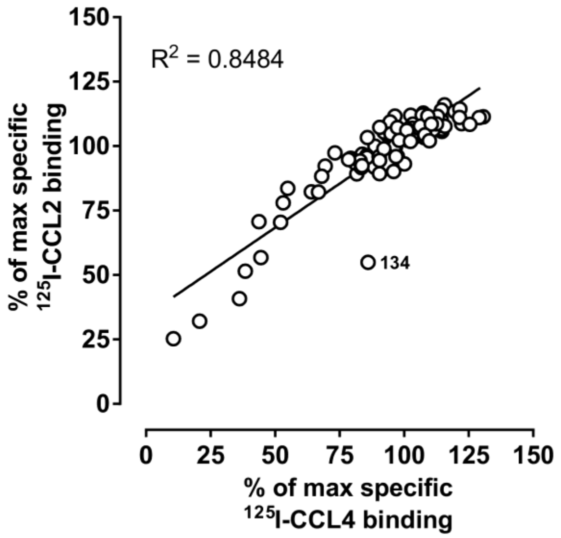

Figure 39. Correlation between ^{125}I-CCL2 and ^{125}I-CCL4 binding displacement by the library of 93 compounds at 10 μM

Out of the five most efficacious CCL2 displacers, three had improved IC_{50} values when compared to VUF2274 (which has an IC_{50} value of 12.35 μM in this experimental setup), but only two were more efficacious than VUF2274: **199** and **208**. Compound **207** was slightly more potent than VUF2274, but could not compete with CCL2 to the same degree, while **134** and **210** had IC_{50} values greater than 10μM (Figure 40).

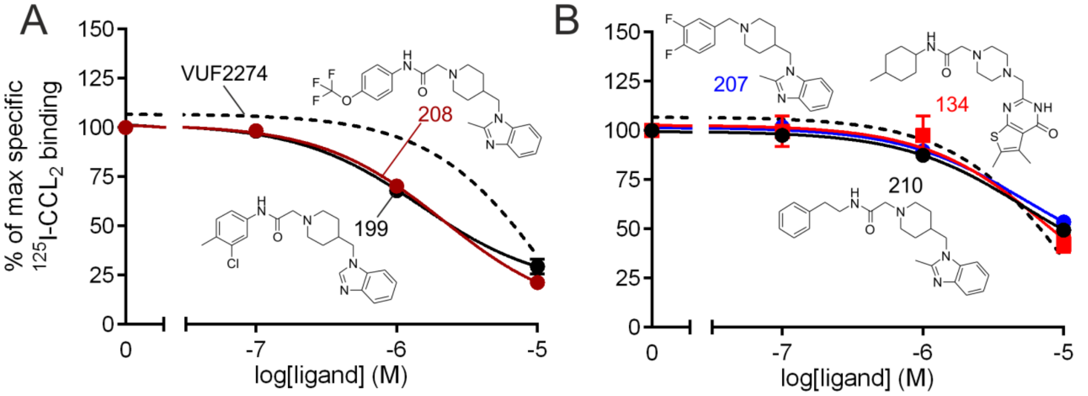

Figure 40. Heterologous competition binding curves against ^{125}I-CCL2 for (A) VUF2274, **199** and **208**; (B) **134, 207** and **210**; data are normalized between the measured radioactivity in the presence of 1 μM unlabeled CCL2 (0%) and 1% DMSO (100%); n=3.

At initial functional evaluation at the US28 wild-type receptor, compounds **199** and **208** behaved as weak agonists, but it was found that their efficacy is dependent on initial experimental conditions. It is interesting that as more constitutive activity is exhibited by the experimental system, the more agonistic these compounds tend to behave. Since this behavior was encountered in other experiments as well, and it was presumed that the underlying cause of this behavior was the rapid constitutive internalization of the US28 receptor, it was decided to also make use of US28Δ300 for compound evaluation, a receptor construct specially tinkered to preserve the constitutive activity of US28, while at the same time eliminating its ability to constitutively internalize due to constitutive β-arrestin2 recruitment. This receptor lacks the last 55 C-terminal amino acids, and possesses no phosphorylation sites for GRKs, and thus cannot recruit arrestins or undergo arrestin-mediated internalization. It has been previously shown that this receptor construct can help identify activity at the US28 receptor which is otherwise 'camouflaged' due to the constitutive internalization of the receptor [47].

Initial screening of all the 93 compounds on the US28Δ300 construct revealed a much more diverse palette of activities than the initial screening performed on the US28WT receptor (Figure 41). Thus, 16 compounds were able to increase IP levels more than 40% over basal and an additional 12 increased US28Δ300 activity by more than 20%. Moreover, 7 compounds lowered basal US28Δ300 IP levels by more than 25%. VUF2274 behaved as a full agonist on US28Δ300, causing an increase in basal activity of over 90%, while **125** had no distinguishable effect on this receptor construct.

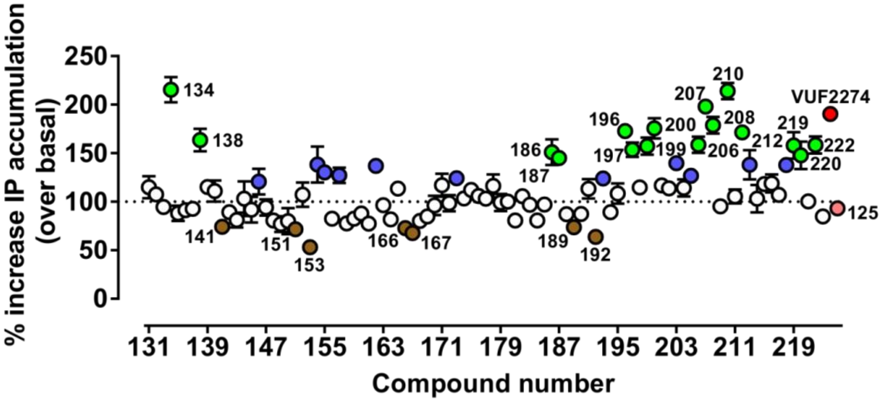

Figure 41. Scatterplot of the initial single dose screening (10 μM) of the 93-compound library in IP accumulation experiments on US28Δ300-expressing HEK-293 cells (n=3);

The compounds capable of potentiating US28Δ300 basal activity by more than 50% had diverse pharmacological behaviors at WT, and therefore no relationship could be established between observed functional behaviors at the two receptor constructs. On the other hand, a relationship was identified between functional behavior at US28Δ300 and chemokine displacement properties (Figure 43), but not between behavior at WT and chemokine displacement properties.

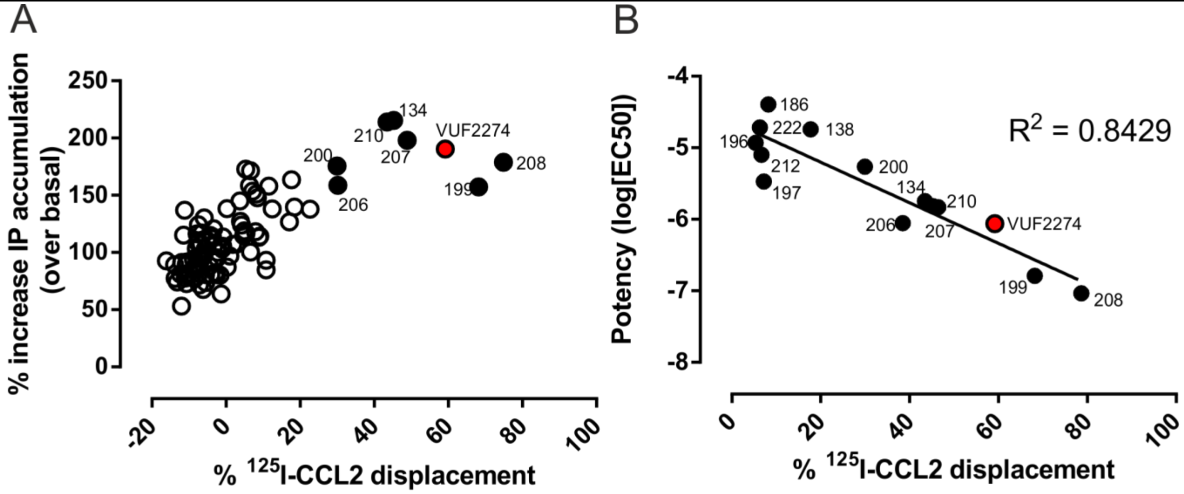

Figure 43. **(A)** Efficacy and **(B)** Potency at US28Δ300 versus ^{125}I-CCL2 displacement efficacy for selected compounds.

Thus, it was demonstrated that activity observed at this receptor construct is camouflaged at WT due to the constitutive internalization properties of US28 and that the use of the US28Δ300 construct can aid in the future identification of novel US28-acting ligands, and is a valuable pharmacological tool for future drug development purposes.

In summary, the main focus of chapter 5 was to identify and characterize novel chemical scaffolds capable of acting at US28, based on the structures of hit compounds described in the previous chapter. Thus, starting from the molecular descriptors of compounds **33**, **125** and **76**, the ZINC database containing >12 million commercially available compounds was screened, and results were ranked, filtered and clustered for the generation of a final second generation library of 93 compound with potential US28 activity.

In vitro assays revealed several novel inverse agonists, out of which **135** and **138** were the most potent, but not particularly efficacious. Compound **205** was 3-fold more potent than parent **125**, with comparable efficacy. In addition, two novel agonists were identified: **199** and **208.**

None of the tested compounds were able to compete with CX_3CL1 for US28 binding to a considerable degree, but several compounds were able to displace CC chemokines from the receptor. At the same time, no relationship between activity at US28WT and chemokine displacement behavior could be established, but a linear dependency between behavior at US28Δ300 and ability to compete with CC chemokines for US28 binding could be observed. This highlights that US28Δ300 is a valuable pharmacological tool for future drug discovery purposes at the US28 receptor. The newly identified scaffolds represent valuable starting points for the generation of novel anti-HCMV therapies targeting the viral-encoded chemokine receptor US28.

General conclusions and perspectives

Understanding the molecular mechanisms behind chemokine receptor activation by small molecule ligands represents the foundation of all drug discovery efforts directed at the chemokine system. In this respect, the research presented herein was directed at:

- describing the molecular mechanisms behind chemokine receptor activation and allosteric modulation by small molecule ligands with a metal center through a combined *in silico* and *in vitro* approach (Chapter 3);
- identifying novel molecular scaffolds able to modulate chemokine receptor activity or influence chemokine binding to receptors, also though a combined *in silico* and *in vitro* approach (Chapters 4 and 5).

In order to fulfill the first of the abovementioned general objectives, research was focused on the chemokine receptor CCR5, which is capable of accommodating several small molecule ligands with a metal center with various pharmacological outcomes. On one hand, the metal-ion complexes ZnBip and ZnTerp are ago-allosteric modulators of this receptor, and therefore can both activate the receptor in the absence of chemokines and modulate chemokine binding to the receptor, while ZnClTerp is a pure allosteric modulator and possesses no intrinsic activity, but strongly impacts chemokine binding to the receptor.

The identification of the binding site for the metal-ion chelator complexes ZnBip, ZnTerp and ZnClTerp was thus pursued, and it was revealed, both through *in silico* and *in vitro* experiments, that the metal-ion chelator binding sites at CCR5 are delineated by residues Y37 and W86 in the minor binding pocket, R168 in ECL2, as well as Y108, F109 and Y251 in the major binding pocket for all three chelator complexes, and additionally for terpyridine complexes ZnTerp and ZnClTerp the major binding pocket residues F112 and W248. At the same time, the increased potency of ZnTerp compared to ZnBip could be explained by the deeper anchorage of ZnTerp in the major binding pocket of the receptor, while the lack of intrinsic activity observed for ZnClTerp could be caused by a faulty interaction of the chelator with W248, the toggle switch tryptophan, which is generally involved in ligand-mediated receptor activation.

In order to fulfill the latter general objective, research was shifted to the viral-encoded chemokine receptor crucial for HCMV pathogenesis, US28, which is closely related to

CCR5. At present, only a limited number of small molecule ligands have been described to act at this receptor, and given the importance of generating novel anti-HCMV-directed therapies due to the danger this virus poses to immunocompromised individuals, it is necessary that new scaffolds capable of modulating the activity of this chemokine receptor are identified.

For this purpose, the ZINC database containing more than 12 million compounds was screened, the results were refined through several filtering steps, and a series of 98 compounds were purchased from commercial vendors and subsequently tested *in vitro*. Out of these, the most potent inverse agonist at US28, as assessed though IP accumulation experiments, was compound **125**, which was capable of lowering 44% of the basal constitutive activity levels of the receptor with an EC_{50} value of 1.76 μM (2-fold improved when compared to parent compound VUF2274 – EC_{50} = 3.5 μM [43]).

It was furthermore found that compound **125** exerts its activity in an allosteric fashion, as it is incapable of competing with all tested chemokines for US28 binding. In fact, out of all the tested compounds in the library, only one was able to compete against radiolabeled CX_3CL1, namely compound **76**, which had no distinguishable activity at US28 WT. However, due to the possible instability of this compound in the biological assays, it remains to be seen if the compound itself or one of its hydrolysis products is responsible for the observed activity.

At the maximum tested concentration (10 μM), compound **125** was more efficacious at lowering the basal constitutive levels of US28 than the endogenous chemokine CX_3CL1 (100 nM). At the same time, compound **33** was identified as an agonist with moderate micromolar potency (EC_{50} = 0.95 μM), and was capable of increasing the basal constitutive activity of US28 by 36% at the maximum tested concentration. It was further established that **33** and **125** share a similar topology, with the right-hand side of the molecule consisting of a bicyclic system linked by a methylene bridge to a central heterocyclic system, piperazine in the case of **33** and piperidine for **125.** Both compounds are accommodated in the US28 binding pocket in a similar fashion, with the central positively charged nitrogen atom of both compounds forming a salt bridge with the chemokine receptor-family conserved glutamic acid in TM VII, E277. The bulky substituents in the right-hand side of the molecules further engage in favorable aromatic interactions with W89 in TM II, Y112 in TM III and Y244 in TM VI.

After identifying at least three potential US28-acting ligands (**33**, **76** and **125**), a new generation of ligands was generated, with the end purpose of identifying novel compounds with improved pharmacological properties compared to their parent compounds. Thus, based on the molecular scaffolds of hits **33**, **76** and **125**, a new library of 93 potential US28-acting compounds was generated and characterized through binding and functional experiments.

Functional evaluation of the 93-compound library revealed some novel inverse agonists superior to parent compound **125** in terms of potency (e.g. **135**, 11-fold more potent, IC_{50} ~ 89 nM), but it was found that the maximal inhibitory effect is dependent on the initial constitutive levels of the experimental system for most of the identified novel inverse agonists. It was furthermore found that none of the tested compounds could displace CX_3CL1 from US28 to a considerable degree. VUF2274 (IC_{50} = 12.35 μM), along with five other compounds (**134**, **199**, **207**, **208** and **210**), were able to displace more than 40% of bound ^{125}I-CCL2. Two of these were more than 5-fold more potent than VU2274 (**199** and **208**). The same two compounds are good displacers of bound ^{125}I-CCL4, as well. VUF2274 could furthermore displace ~65% of bound ^{125}I-CCL4. At the same time, **125** could not compete with any of the tested chemokines for US28 binding.

Thus, it was found that compounds which share the same chemical scaffold with parent compound **125** are able to compete with CCL2 and CCL4 but not CX_3CL1 for US28 binding in order to generate various functional outcomes. To the present day, this is the first report describing small molecule ligands capable of competing with inflammatory chemokines for HCMV-encoded US28 receptor binding, other than CCL5, and at the same time is the first report of preferential chemokine displacement for small molecules at US28.

No direct link between chemokine displacement and functional activity at US28WT was found, but a correlation between ^{125}I-CCL2 displacement and activity at a receptor construct devoid of β-arrestin-mediated internalization and endocytosis, US28Δ300, was identified. It is postulated that the activity observed at this receptor construct is camouflaged at US28WT due to the constitutive internalization properties of the wild-type viral-encoded chemokine receptor and that the use of this receptor construct could aid in the future identification of novel US28-acting ligands.

Further research will focus on improving the pharmacological properties of the novel scaffolds with US28 activity identified during this thesis, with the end purpose of generating improved anti-HCMV therapies. At the same time, the knowledge and computational tools presented in the thesis will be transferred to other GPCR systems in order to accelerate the discovery of novel ligands or molecular scaffolds, especially in the case of receptors which have been particularly challenging to study from a drug development point of view due to the scarcity of specific small molecule ligands.

Personal contributions

Parts of the results presented in the thesis are the subject of several research articles, as well as a book chapter, in addition to have been presented at national and international conferences in the form of posters or short communications.

Publications:

1. Karlshøj S, **Amarandi R-M**, Larsen O, Daugvilaite V, Steen A, Brvar M, Pui A, Frimurer TM, Ulven T, Rosenkilde MM (2016) Molecular Mechanism of Action for Allosteric Modulators and Agonists in CC-chemokine Receptor 5 (CCR5), *Journal of Biological Chemistry* **291**(52):26860-26874 (DOI: 10.1074/jbc.M116.740183), **IF = 4.258** [48].
2. Luckmann M[⊥], **Amarandi R-M**[⊥], Papargyri N, Jakobsen MH, Christiansen E, Jensen LJ, Pui A, Schwartz TW, Rosenkilde MM, Frimurer TM (2017) Structure-based Discovery of Novel US28 Small Molecule Ligands with Different Modes of Action, *Chemical Biology & Drug Design* **89**(3):289-296 (DOI: 10.1111/cbdd.12848), **IF = 2.802** [49].
3. **Amarandi R-M**[⊥], Luckmann M[⊥], Melynis M, Frimurer TM, Jakobsen MH, Frimurer TM, Rosenkilde MM – Probe dependence in allostery: the case of small molecule ligands acting on HCMV-encoded chemokine receptor US28 (manuscript in preparation)
4. **Amarandi R-M**, Hjortø GM, Rosenkilde MM, Karlshøj S (2016) Probing Biased Signaling at Chemokine Receptors, in Handel TM (Ed.), *Methods in Enzymology Vol. 570 – Chemokines*, Academic Press, Cambridge MA, pp.155-186, indexed in Thomson Reuters Web of Knowledge (DOI: 10.1016/bs.mie.2015.09.001) [50].

Total impact factor: **4.258 + 2.802 = 7.06**

Conferences:

1. **Amarandi R-M**, Karlshøj S, Rosenkilde MM, Pui A, "Structural insights into the zinc-mediated activity of polypyridine chelators at the chemokine receptor CCR5", XVIIIth International Conference 'Physical Methods in Coordination and Supramolecular Chemistry', Chișinău (Moldova), 8-9th of October 2015 – poster

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[⊥] Shared first authorship

2. **Amarandi R-M**, Karlshøj S, Rosenkilde MM, Pui A "Molecular determinants of polypyridine chelator activity at the chemokine receptor CCR5 in the presence of Zn(II) salts", "Alexandru Ioan Cuza" University days, Faculty of Chemistry Conference, Iași (România), 29th-31st of October 2015 – oral communication

Traineeship periods:

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2. **05/2015 – 09/2015** – LLP-Erasmus Student Mobility for Placement, Institute of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark
3. **05/2014 – 08/2014** – LLP-Erasmus Student Mobility for Placement, Institute of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark

Selected references

[1] Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (**2000**) Crystal structure of rhodopsin: A G protein-coupled receptor, *Science* **289**:739-45.

[2] Drews J (**2000**) Drug discovery: a historical perspective, *Science* **287**:1960-4.

[3] Liman ER (**2006**) Use it or lose it: molecular evolution of sensory signaling in primates, *Pflugers Arch* **453**:125-31.

[4] Pan H-L, Wu Z-Z, Zhou H-Y, Chen S-R, Zhang H-M, Li D-P (**2008**) Modulation of Pain Transmission by G Protein-Coupled Receptors, *Pharmacology & Therapeutics* **117**:141-61.

[5] Kobayashi NR, Hawes SM, Crook JM, Pebay A (**2010**) G-protein coupled receptors in stem cell self-renewal and differentiation, *Stem Cell Rev* **6**:351-66.

[6] Betke KM, Wells CA, Hamm HE (**2012**) GPCR Mediated Regulation of Synaptic Transmission, *Progress in Neurobiology* **96**:304-21.

[7] Filmore D (**2004**) It'sa GPCR world, *Modern drug discovery* **7**:24-8.

[8] Jacobson KA (**2015**) New paradigms in GPCR drug discovery, *Biochemical pharmacology* **98**:541-55.

[9] Deupi X, Kobilka B (**2007**) Activation of G protein–coupled receptors, *Advances in protein chemistry* **74**:137-66.

[10] Schwartz TW, Rosenkilde MM (**1996**) Is there a 'lock' for all agonist 'keys' in 7TM receptors?, *Trends Pharmacol Sci* **17**:213-6.

[11] Zhang R, Xie X (**2012**) Tools for GPCR drug discovery, *Acta Pharmacologica Sinica* **33**:372-84.

[12] Elling CE, Thirstrup K, Nielsen SM, Hjorth SA, Schwartz TW (**1997**) Metal-ion sites as structural and functional probes of helix-helix interactions in 7TM receptors, *Ann N Y Acad Sci* **814**:142-51.

[13] Elling CE, Thirstrup K, Nielsen SM, Hjorth SA, Schwartz TW (**1997**) Engineering of metal-ion sites as distance constraints in structural and functional analysis of 7TM receptors, *Fold Des* **2**:S76-80.

[14] Rosenkilde MM, Lucibello M, Holst B, Schwartz TW (**1998**) Natural agonist enhancing bis-His zinc-site in transmembrane segment V of the tachykinin NK3 receptor, *FEBS Lett* **439**:35-40.

[15] Elling CE, Thirstrup K, Holst B, Schwartz TW (**1999**) Conversion of agonist site to metal-ion chelator site in the beta(2)-adrenergic receptor, *Proc Natl Acad Sci U S A* **96**:12322-7.

[16] Rosenkilde MM, David R, Oerlecke I, Benned-Jensen T, Geumann U, Beck-Sickinger AG, Schwartz TW (**2006**) Conformational constraining of inactive and active States of a seven transmembrane receptor by metal ion site engineering in the extracellular end of transmembrane segment V, *Mol Pharmacol* **70**:1892-901.

[17] Rosenkilde MM, Andersen MB, Nygaard R, Frimurer TM, Schwartz TW (**2007**) Activation of the CXCR3 chemokine receptor through anchoring of a small molecule chelator ligand between TM-III, -IV, and -VI, *Mol Pharmacol* **71**:930-41.

[18] Sheikh SP, Zvyaga TA, Lichtarge O, Sakmar TP, Bourne HR (**1996**) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F, *Nature* **383**:347-50.

[19] Sheikh SP, Vilardarga JP, Baranski TJ, Lichtarge O, Iiri T, Meng EC, Nissenson RA, Bourne HR (**1999**) Similar structures and shared switch mechanisms of the beta2-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation, *J Biol Chem* **274**:17033-41.

[20] Jensen PC, Thiele S, Ulven T, Schwartz TW, Rosenkilde MM (**2008**) Positive versus negative modulation of different endogenous chemokines for CC-chemokine receptor 1 by small molecule agonists through allosteric versus orthosteric binding, *J Biol Chem* **283**:23121-8.

[21] Thiele S, Steen A, Jensen PC, Mokrosinski J, Frimurer TM, Rosenkilde MM (**2011**) Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach, *J Biol Chem* **286**:37543-54.

[22] Thiele S, Malmgaard-Clausen M, Engel-Andreasen J, Steen A, Rummel PC, Nielsen MC, Gloriam DE, Frimurer TM, Ulven T, Rosenkilde MM (**2012**) Modulation in selectivity and allosteric properties of small-molecule ligands for CC-chemokine receptors., *J Med Chem* **55**:8164-77.

[23] Yoshie O (**2013**) Chemokine receptors as therapeutic targets, *Nihon Rinsho Meneki Gakkai Kaishi* **36**:189-96.

[24] Boppana SB, Ross SA, Fowler KB (**2013**) Congenital Cytomegalovirus Infection: Clinical Outcome, *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* **57**:S178-S81.

[25] Centers for Disease and Control Prevention (**2016)** Babies Born with CMV (Congenital CMV Infection), accesesed 6 March 2017, retrieved from: https://www.cdc.gov/cmv/congenital-infection.html

[26] Pease JE, Horuk R (**2015**) Chemokine Receptors in Allergy, Inflammation, and Infectious Disease, in: Tschammer N (Ed.) *Chemokines: Chemokines and Their Receptors in Drug Discovery*, Springer International Publishing, Cham, pp. 1-39.

[27] Chalikiopoulos A, Thiele S, Malmgaard-Clausen M, Rydberg P, Isberg V, Ulven T, Frimurer TM, Rosenkilde MM, Gloriam DE (**2013**) Structure-activity relationships and identification of optmized CC-chemokine receptor CCR1, 5, and 8 metal-ion chelators, *J Chem Inf Model* **53**:2863-73.

[28] Rosenkilde MM, Kledal TN, Brauner-Osborne H, Schwartz TW (**1999**) Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74, *J Biol Chem* **274**:956-61.

[29] Schwartz TW, Frimurer TM, Holst B, Rosenkilde MM, Elling CE (**2006**) Molecular mechanism of 7TM receptor activation--a global toggle switch model, *Annu Rev Pharmacol Toxicol* **46**:481-519.

[30] Khan A, Nicholson G, Greenman J, Madden L, McRobbie G, Pannecouque C, De Clercq E, Ullom R, Maples DL, Maples RL, Silversides JD, Hubin TJ, Archibald SJ (**2009**) Binding optimization through coordination chemistry: CXCR4 chemokine receptor antagonists from ultra rigid metal complexes, *J Am Chem Soc* **131**:3416-7.

[31] De Clercq E (**2009**) The AMD3100 story: the path to the discovery of a stem cell mobilizer (Mozobil), *Biochem Pharmacol* **77**:1655-64.

[32] Gerlach LO, Skerlj RT, Bridger GJ, Schwartz TW (**2001**) Molecular interactions of cyclam and bicyclam non-peptide antagonists with the CXCR4 chemokine receptor, *J Biol Chem* **276**:14153-60.

[33] Kufareva I, Abagyan R, Handel TM (**2015**) Role of 3D Structures in Understanding, Predicting, and Designing Molecular Interactions in the Chemokine Receptor Family, in: Tschammer N (Ed.) *Chemokines: Chemokines and Their Receptors in Drug Discovery*, Springer International Publishing, Cham, pp. 41-85.

[34] Vomaske J, Nelson JA, Streblow DN (**2009**) Human Cytomegalovirus US28: a functionally selective chemokine binding receptor, *Infect Disord Drug Targets* **9**:548-56.

[35] Wills MR, Poole E, Lau B, Krishna B, Sinclair JH (**2015**) The immunology of human cytomegalovirus latency: could latent infection be cleared by novel immunotherapeutic strategies?, *Cell Mol Immunol* **12**:128-38.

[36] Casarosa P, Bakker RA, Verzijl D, Navis M, Timmerman H, Leurs R, Smit MJ (**2001**) Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28., *The journal of biological chemistry* **276**:1133-7.

[37] Vischer HF, Hulshof JW, de Esch IJ, Smit MJ, Leurs R (**2006**) Virus-encoded G-protein-coupled receptors: constitutively active (dys)regulators of cell function and their potential as drug target, *Ernst Schering Found Symp Proc* 187-209.

[38] Miller WE, Zagorski WA, Brenneman JD, Avery D, Miller JL, O'Connor CM (**2012**) US28 is a potent activator of phospholipase C during HCMV infection of clinically relevant target cells, *PLoS One* **7**:e50524.

[39] Langemeijer EV, Slinger E, de Munnik S, Schreiber A, Maussang D, Vischer H, Verkaar F, Leurs R, Siderius M, Smit MJ (**2012**) Constitutive beta-catenin signaling by the viral chemokine receptor US28, *PLoS One* **7**:e48935.

[40] Kledal TN, Rosenkilde MM, Schwartz TW (**1998**) Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28, *FEBS Lett* **441**:209-14.

[41] Spiess K, Jeppesen MG, Malmgaard-Clausen M, Krzywkowski K, Dulal K, Cheng T, Hjorto GM, Larsen O, Burg JS, Jarvis MA, Garcia KC, Zhu H, Kledal TN, Rosenkilde MM (**2015**) Rationally designed chemokine-based toxin targeting the viral G protein-coupled receptor US28 potently inhibits cytomegalovirus infection in vivo, *Proc Natl Acad Sci U S A* **112**:8427-32.

[42] Krishna BA, Spiess K, Poole EL, Lau B, Voigt S, Kledal TN, Rosenkilde MM, Sinclair JH (**2017**) Targeting the latent cytomegalovirus reservoir with an antiviral fusion toxin protein, *Nat Commun* **8**:14321.

[43] Casarosa P, Menge WM, Minisini R, Otto C, van Heteren J, Jongejan A, Timmerman H, Moepps B, Kirchhoff F, Mertens T, Smit MJ, Leurs R (**2003**) Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor, *J Biol Chem* **278**:5172-8.

[44] Hesselgesser J, Ng HP, Liang M, Zheng W, May K, Bauman JG, Monahan S, Islam I, Wei GP, Ghannam A, Taub DD, Rosser M, Snider RM, Morrissey MM, Perez HD, Horuk R (**1998**) Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor, *J Biol Chem* **273**:15687-92.

[45] Lee S, Chung YH, Lee C (**2017**) US28, a Virally-Encoded GPCR as an Antiviral Target for Human Cytomegalovirus Infection, *Biomolecules & Therapeutics* **25**:69-79.

[46] Irwin JJ, Sterling T, Mysinger MM, Bolstad ES, Coleman RG (**2012**) ZINC: a free tool to discover chemistry for biology, *J Chem Inf Model* **52**:1757-68.

[47] Tschammer N (**2014**) Allosteric modulation of the G protein-coupled US28 receptor of human cytomegalovirus: are the small-weight inverse agonist of US28 'camouflaged' agonists?, *Bioorg Med Chem Lett* **24**:3744-7.

[48] Karlshoj S, Amarandi RM, Larsen O, Daugvilaite V, Steen A, Brvar M, Pui A, Frimurer TM, Ulven T, Rosenkilde MM (**2016**) Molecular Mechanism of Action for Allosteric Modulators and Agonists in CC-chemokine Receptor 5 (CCR5), *J Biol Chem* **291**:26860-74.

[49] Lückmann M, Amarandi R-M, Papargyri N, Jakobsen MH, Christiansen E, Jensen LJ, Pui A, Schwartz TW, Rosenkilde MM, Frimurer TM (**2017**) Structure-based discovery of novel US28 small molecule ligands with different modes of action, *Chemical Biology & Drug Design* **89**:289-96.

[50] Amarandi R-M, Hjortø GM, Rosenkilde MM, Karlshøj S (**2016**) Probing Biased Signaling in Chemokine Receptors, in: Handel TM (Ed.) *Methods in enzymology*, **570**, Academic Press, Cambridge, MA, pp. 155-86.