Design and synthesis of small nonpeptidergic ligands for the human cytomegalovirus-encoded receptor US28

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Introduction

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Contents

Chemokines and chemokine receptors

The G protein-coupled receptor (GPCR) family has emerged as the most important target for therapeutic intervention, as currently more than 60% of all known marketed drugs target GPCRs.[1] Most of these small-molecule ligands are acting on class A biogenic amine receptors, such as the serotonin, histamine, adrenoceptors, and the muscarinic acetylcholine and dopamine receptors. Currently, also small molecules acting on other classes of the GPCR family are developed, especially those of the peptide or protein binding receptor classes.

The chemokine receptor family is the largest subfamily of peptide-binding GPCRs described until now (Figure 1).[2] Chemokine receptors play an important role in several disease processes, e.g. in cancer,[3] acute and chronic inflammation, angiogenesis and angiostasis, and as co-receptors for the cellular entry of human immunodeficiency virus (HIV).[2] These receptors are defined by their ability to signal on binding one or more members of the chemokine superfamily of small (8-14 kDa) chemotactic cytokines.

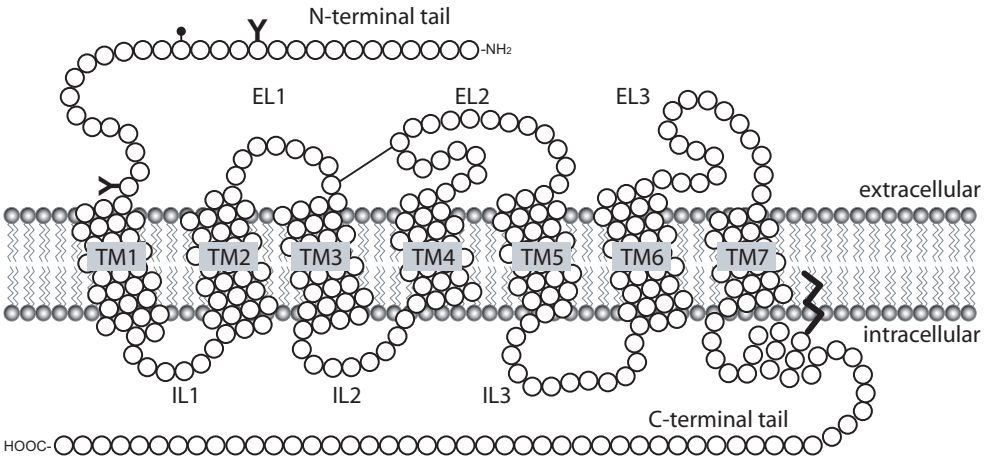


Figure 1. Topology of a chemokine receptor. The separate amino acids are shown as circles. The *N*-terminus and the extracellular loops (EL) are located outside the cell, while the *C*-terminus resides intracellularly. The transmembrane regions (TM) are located in the membrane of the cell. Adapted from Fernandez et al.[4]

Chemokines are classified into two major (CXC and CC) and two minor (C and CX3C) groups, dependent on the number and spacing of conserved cysteine residues in their amino terminus (Table 1 and 2).[5] CXC, CC, and CX3C chemokines all have four conserved cysteine residues, whereas C chemokines

have only two, corresponding to the second and fourth cysteines in the other groups.[6] CXC and CX3C chemokines are distinguished by the presence of one (CXC) or three (CX3C) amino acids between the first and second cysteine residues, whereas the first two cysteines in CC chemokines are adjacent. All chemokines consist of three beta sheets and an alpha helix, which separate the short *N*-terminus and the *C*-terminal domains. Interestingly, the different chemokines adopt a similar tertiary folding, even in cases of low overall sequence identity (varying from 20 to 95%).[6]

Table 1. Overview of the CC chemokine receptors and their ligands.[2,7]

CC Family of Chemokines and Chemokine Receptors		
Receptor	**Chemokine ligands**	**Associated diseases**
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC1)	Rheumatoid arthritis, multiple sclerosis, allograft rejection
CCR2	CCL2 (MCP-1), CCL8 (MCP-2), CCL7, CCL13 (MCP-4), CCL16 (HCC4)	Atherosclerosis, rheumatoid arthritis, multiple sclerosis, asthma, resistance to intracellular pathogens, type 2 diabetes mellitus
CCR3	CCL5, CCL7, CCL8, CCL11 (eotaxin), (MCP-2), CCL13, CCL24	Allergic asthma, rhinitis, contact dermatitis
CCR4	CCL17 (TARC), CCL22 (MDC)	Parasitic infection, graft rejection, asthma, contact dermatitis, sepsis
CCR5	CCL3, CCL4 (MIP-1β), CCL5, CCL11, CCL14, CCL16	HIV-1 co-receptor, allograft rejection, multiple sclerosis, rheumatoid arthritis
CCR6	CCL20 (MIP-3β, LARC)	Mucosal humoral immunity, allergic asthma, psoriasis
CCR7	CCL19 (ELC), CCL21 (SLC)	Cancer
CCR8	CCL1 (1309)	Atopic dermatitis, asthma
CCR9	CCL25 (TECK)	Intestinal inflammation, inflammatory bowel disease
CCR10	CCL27 (CTACK), CCL28 (MEC)	Skin inflammation and endothelium, dermal skin and colon ulcerative colitis.

Currently, approximately 45 chemokines and 19 chemokine receptors have been identified in humans.[8] These numbers are still growing, because chemokines exist for which the receptor has not yet been identified. Morover, there are several protein sequences with high homology to chemokine receptors for which the ligand is unknown and are thus considered as orphan receptors.[9]

Binding of chemokines to their cognate receptors is remarkable promiscuous and redundant. Most chemokine receptors can bind more than one chemokine and

most chemokines can activate several different chemokine receptors.[10] However, binding to chemokine receptors is almost always restricted to a single subclass of chemokines, so CC chemokine receptors can only be activated by CC chemokines, while CXC chemokines only bind to CXC chemokine receptors.[6] Only the promiscuous chemokine binding proteins D6 and DARC (Duffy antigen receptor for chemokines) and the human cytomegalovirus (HCMV) encoded receptor US28 have been shown to bind both CC and CXC chemokines with equal affinity.[11-15]

Table 2. Overview of the CXC, CX3C and XC chemokine receptors and their ligands.[2,7]

CXC, CX3C and XC Families of Chemokines and Chemokine Receptors		
Receptor	**Chemokine ligands**	**Associated disease**
CXCR1	CXCL8 (interleukin-8), CXCL6 (GCP2)	Inflammatory lung disease, COPD (chronic obstructive pulmonary disease), psoriasis, cancer
CXCR2	CXCL8, CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6	Inflammatory lung disease, COPD, psoriasis, cancer, atherosclerosis
CXCR3-A	CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)	Inflammatory skin disease, multiple sclerosis, allograft rejection
CXCR3-B	CXCL4 (PF4), CXCL9, CXCL10, CXCL11	Cancer
CXCR4	CXCL12 (SDF-1)	HIV-1 co-receptor, cancer
CXCR5	CXCL13 (BCA-1)	Cancer
CXCR6	CXCL16 (SR-PSOX)	Inflammatory liver diseas, atherosclerosis
CXCR7[16]	CXCL11, CXCL12	Cancer, HIV-1 co-receptor
CX3CR1	CX3CL1 (fractalkine)	Atherosclerosis, allograft rejection, CNS (central nervous system) inflammation
XCR1	CXL1 (lymphotactin), XCL2	Rheumatoid arthritis, nephropathy, cancer

The main role of chemokines is the recruitment of leukocytes, thereby playing a key role in the immune system.[9,17] There are two different types of chemokines. Constitutive chemokines are responsible for the basal leukocyte trafficking and the development of normal immune responses, for example in the trafficking of lymphocytes to lymphoid tissues. These chemokines are homeostatic in nature and are constitutively produced. In contrast, inducible or inflammatory chemokines are only secreted during infection or a pro-inflammatory stimulus. These chemokines regulate the migration of leukocytes to sites of injury or

infection, activate cells to mount an immune response or initiate wound healing.[4]

Inappropriate expression of certain chemokines can lead to excessive leukocyte recruitment and activation. If the immune response is inappropriately activated and targeted towards normal healthy tissue, this results in autoimmunity and disease. Chemokines are suggested to play a role in cancer and a number of inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, asthma and organ transplant rejection (Table 1 and 2). Based on the potential role of chemokines and chemokine receptors in the pathophysiology of these diseases, chemokine receptor antagonists are considered as interesting therapeutics for the treatment of these and other proinflammatory diseases.[2] As a consequence, a number of companies have initiated drug finding programs to discover chemokine receptor antagonists that could validate the potential of chemokine receptors as drug targets.

A very important discovery in the chemokine field was the identification of chemokine receptors as co-receptors for HIV-1 entry into host cells.[18] Human and simian immunodeficiency viruses (HIV and SIV) require a chemokine receptor in addition to CD4 for efficient entry into cells. HIV enters cells by binding to CD4 on the cell surface of the host.[19] This induces a conformational change in the envelope surface glycoprotein gp120 that allows a secondary interaction with a chemokine receptor, thereby acting as a co-receptor for HIV. Further structural changes in the envelope transmembrane protein gp41 result in membrane fusion and virus entry in the host cell. CCR5 and CXCR4 are the most important co-receptors for HIV, but a large number of other seven transmembrane (7TM) GPCRs have been shown to act as co-receptors as well, including CC, CXC and CX3C chemokine receptors as well as orphan and viral-encoded GPCRs.[20]

The most relevant co-receptor in HIV infection appears to be CCR5, since individuals with a defective CCR5 allele exhibit resistance to HIV-1 infection. This mutant allele of CCR5, CCR5-Δ32, is frequent in populations of Caucasian origin (about 1% of the population), but was not found in native African, American

Indian, and East Asian ethnic groups.[21] Individuals homozygous for CCR5-Δ32 cannot produce a complete CCR5 chemokine receptor, resulting in a non-functional truncated protein that is not transported to the cell surface. Thus, the HIV-1 virus cannot attach to the host cells and subsequently enter the cytosol resulting in nearly resistance to HIV-1 infection despite repeated exposure. Heterozygous individuals of the CCR5-Δ32 (about 20% of the population) express less functional receptors on the cell surface and display delayed progression of 2-3 years to acquired immunodeficiency syndrome (AIDS).[21,22] In heterozygous carriers the mutant CCR5-Δ32 receptor heterocomplexes with normal CCR5, so there is only half the amount of the normal CCR5 allele present at the cell surface. Moreover, the mutated CCR5-Δ32 seems to reduce the expression of the normal allele at the cell surface by keeping CCR5 in the endoplasmic reticulum. Thus, normal CCR5 can no longer mediate HIV-1 infection, which can explain the delayed onset of AIDS in CCR5/CCR5-Δ32 individuals.[23]

In conclusion, the finding that some chemokines and their receptors are upregulated in both acute and chronic inflammatory diseases, and the important role that they play in the development of AIDS, made them new interesting drug targets for therapeutic intervention in these diseases.[24]

Viral hijacking of the chemokine system

Viruses exploit the host cell to guarantee their replication and survival. In order to take over control from the cell, viruses unleash several strategies upon infection. In one of these strategies, host proteins from the chemokine system are mimicked to take control of key processes. Several members of the large DNA virus families, namely herpesviruses and poxviruses, have incorporated chemokines and/or chemokine receptors from their hosts into their own viral genome to affect chemokine signaling for their own benefit.[25]

Currently, there are more than 50 different viral chemokines, chemokine binding proteins or chemokine receptors identified in various herpesviruses, poxviruses and retroviruses (Figure 2).

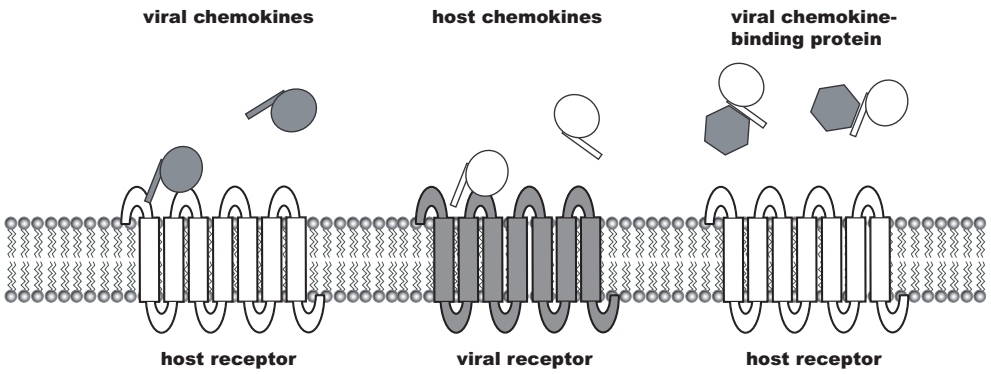


Figure 2. Three mechanism by which viruses can exploit the host chemokine system. Left: the viral-encoded chemokines that are able to bind to endogenous chemokine receptors. Middle: the viral-encoded chemokine receptors. Right: the virus-encoded chemokine-binding proteins (vCKBPs) that act as chemokine antagonists. Adapted from Rosenkilde et al.[26]

Viral chemokines have presumably been acquired through an ancient act of molecular piracy from the host genome. It is suggested that these chemokine mimics are of biological importance for the viral life cycle, because of the high conservation throughout different viral strains.[26] They are also suggested to play a role in the subversion of the host immune response or the dysregulation of cell growth.[27] Viral-encoded chemokines show a high degree of complexity in their pharmacological profile. They can act selectively on one particular chemokine receptor, e.g. MC148 encoded by the poxvirus Molluscum cantagiosum is a selective antagonist for the mammalian chemokine receptor CCR8.[28] On the other hand, a promiscuous binding profile by acting on different chemokine receptors is shown for vMIP-2, a CC chemokine encoded by human herpesvirus 8 (HHV-8), that acts as an antagonist on numerous endogenous and viral-encoded CC and CXC chemokine receptors.[29] Moreover, virus-encoded chemokines can have different pharmacological properties on chemokine receptors ranging from agonism, antagonism to even inverse agonism.[26]

Viral-encoded chemokine binding proteins (vCKBPs) are encoded by members of the poxviruses and the herpesvirus murine γ-herpesvirus 68 (MHV68). These viral chemokine inhibitors are secreted proteins that bind to a broad range of chemokines and function as chemokine scavengers.[26,30] The vCKBPs are not found in the mammalian chemokine system, so they represent an interesting class of anti-inflammatory chemokine modulators generated by the virus as

antagonists of the endogenous chemokine system. Currently, there are three classes of vCKBPs. M-T7, encoded by Myxoma virus, is the only representative of the vCKBP1 class. This vCKBP is a low affinity binder of a broad range of chemokines at their heparin binding site, thereby inhibiting chemokine action by preventing the interaction of these chemokines with the glycosaminoglycan (GAG) binding site.[31] The vCKBP2 class consists of different chemokine binding proteins encoded by poxviruses, which selectively antagonize the action of CC chemokines by binding at their receptor binding site.[32] To date, M3, encoded by murine herpesvirus 68 (MHV68), is the only member of the vCKBP3 group. This protein is a broad-spectrum chemokine antagonists and inhibits all four classes of chemokines by binding at both the receptor binding site as well as the GAG binding site.[33]

Another strategy used by several large DNA herpesviruses and lentiviruses to exploit the host cell is chemokine mimicry.[34] Probably the best-known employment of GPCRs by viruses is the use of several mammalian chemokine receptors as HIV entry factors.[35] Yet, viral homologues of chemokine receptors have also been identified in several viruses, among which are human, murine and rat cytomegalovirus, as well as HHV-6, HHV-7 and the γ-herpesvirus HHV-8. Only a couple of these viral GPCRs have so far been demonstrated to be functional chemokine receptors with respect to signal transduction and chemokine binding properties.[25]

Although more and more information on the signal transduction pathways and chemokine binding properties of viral-encoded GPCRs is elucidated, their exact role during the viral life cycle and viral pathogenesis is not known yet. GPCRs have key roles in cellular communication and chemokine receptors are important in the regulation of the immune system and other important pathophysiological conditions. Thus, vGPCRs could play an important role in viral action.[36] It can be hypothesized that the signaling capacity and chemokine binding profile of viral-encoded GPCRs have been optimized through an evolutionary selection in favour of viral survival and replication. This results in immune evasion by acting as scavengers of endogenous chemokines through rapid endocytosis and reprogramming of the cellular machinery to help the virus in various parts of the

viral life cycle.[37] Additional potential roles are cellular activation, tissue targeting and cell entry.[25] In contrast to their mammalian homologues, a number of viral-encoded chemokine receptors activate different signaling pathways in a ligand-independent manner. This constitutive signaling of many vGPCRs and the present knowledge that chemokines and their receptors are associated with inflammatory diseases and tumour metastasis[38] suggests that viral-encoded chemokine receptors play an important role in virus-associated diseases.[37]
In summary, it has been demonstrated that chemokine mimicry is common and important for the pathology of various herpes- and poxvirus infections, but the reason why has not been elucidated yet. Nevertheless, viral mimicry of chemokines and chemokine receptors provides us with interesting and challenging putative new drug targets for a novel class of anti-viral therapy.

Human cytomegalovirus

Human cytomegalovirus (HCMV) or human herpesvirus-5 (HHV-5) is a highly species-specific β-herpesvirus that establishes lifelong latency in the host.[39] The virus infects endothelial, epithelial, and smooth muscle cells of the upper gastrointestinal, respiratory, and urogenital tract,[40] and is spread throughout the body by latently infected monocytes.[41] Differentiation of these monocytes into macrophages is accompanied by reactivation of CMV leading to the release of infectious virions. [41] Infection of individuals with HCMV is common and arises progressively from early age, reaching a seroprevalence of 30-70% in developed countries.[42] However, in poor socioeconomic groups, homosexuals and among people in developing countries the seroprevalence can reach up to 90% in adults.[43] The highest infection rates are observed in adolescents and in young children attending group day care, where CMV shedding and transmission are common.[44] HCMV can be transmitted via saliva, sexual contact, placental transfer, breastfeeding, blood transfusion, solid-organ transplantation or haematopoietic stem-cell transplantation.[46]

Primary infection in healthy individuals rarely causes a serious illness, but may be associated with a mononucleosis-like syndrome. In contrast, the primary infection or reactivation of the virus in immunocompromised hosts, such as premature neonates, transplant recipients, or HIV-infected people, can cause

serious and even life-threatening conditions.[39,45] One of the most important clinical manifestations of primary HCMV infection is seen in newborn babies, of which the mothers are infected during pregnancy.[46] It is estimated that congenital CMV transmission occurs in 0.5–2% of all newborns.[47] The clinical symptoms of congenital CMV infection are quite variable, varying from hearing defects or neurodevelopmental problems to irreversible CNS involvement in the form of microcephaly, encephalitis, seizures, deafness, upper motor neuron disorders, psychomotor retardation or blindness.[46] Long-term follow up studies revealed that up to 80% of the affected infants display serious life-long neurological abnormalities with severe life-threatening organ dysfunction and death in 10–20% of patients.

Infection with HCMV is furthermore suggested to be associated with vascular diseases, such as arterial restenosis, atherosclerosis and chronic allograft rejection,[48-51] and increasing evidence indicates that CMV may also contribute to inflammatory and autoimmune diseases[52] as well as colon cancer[53] and malignant glioma.[54] After bone marrow or hematopoietic stem cell transplantations, pneumonia and enteritis are the most common clinical manifestations of HCMV disease. However, in solid-organ transplant recipients, infection with HCMV has been linked to indirect effects, such as dysfunction or rejection of the transplanted organ, an increased risk for bacterial or fungal opportunistic infections, and accelerated atherosclerosis in heart transplant recipients.[55] Moreover, co-infection of human cytomegalovirus with HIV has been shown to cause an increased risk of disease progression to AIDS and dementia in HIV patients.[56]

The HCMV-encoded receptor US28 as a potential anti-viral drug target?

The genome of HCMV encodes at least four GPCRs, which are encoded by the open reading frames (ORFs) UL33, UL78, US27, and US28.[57] A recent study suggests that 11 additional ORFs might encode proteins with seven transmembrane regions, but none of these genes seem to have other sequence characteristics of members of known GPCR families.[58] Recently, it was confirmed that cytomegaloviruses of higher primates, including HCMV, encode a complete novel family of GPCRs in their genome, namely the US12 family, which consists

of the ten contiguous genes US12 through US21.[59] These viral genes are not essential for viral replication in vitro, but it is suggested that they play important roles in HCMV biology.

UL33 and UL78 both have counterparts in the genome of all sequenced β-herpesviruses, including rat CMV (R33 and R78, respectively) and mouse CMV (M33 and M78, respectively). The genes of US27 and US28 are located in the unique short (US) region of the genome of CMV, which is only present in primate CMV, namely in human, chimpanzee, African green monkey and rhesus macaque CMV.[37] UL33, UL78, US28 and presumably US27 are incorporated in the viral envelope,[60-64] but expression of these proteins is not essential for viral replication in vitro.[60,65-69] At present, no chemokines are known to bind to UL33, UL78 or US27, so these receptors remain orphan receptors. UL33 shows highest amino acid sequence identity to the CC chemokine receptors CCR3 and CCR10, while US27 displays a 23% sequence identity to CXCR3.[37] UL78 has a limited sequence identity to chemokine receptors or other GPCRs, and only shares some general conserved GPCR features.[70] UL33 constitutively activates multiple signaling pathways through various G proteins in both transfected as well as infected cells, while no signaling has been found yet for both UL78 and US27.[71,72]

Although the exact roles of UL33 and UL78 are not known yet, in vivo experiments clearly illustrate the significance of the UL33 and UL78 family members in the pathogenesis of CMV infection. The biological significance of R33 and M33 has been shown for recombinant rat and murine CMV strains in which either the R33 or the M33 genes were deleted. In contrast to their wild type counterparts, these mutant viruses were unable to replicate in salivary glands and caused a lower mortality rate in infected animals.[65,73] Additionally, the R33-deleted mutant virus showed a reduced RCMV accelerated transplant vascular sclerosis and chronic rejection in a rat heart transplantation/chronic rejection model.[74] Mutant viruses were also generated for the UL78 family members. Disruption of the UL78 gene of HCMV did not influence viral replication in vitro.[69] In contrast, in vivo experiments showed that deletion of the R78 gene from the genome of rat CMV resulted in a lower replication rate in spleen,[75] while

replication of the M78-deleted mutant virus was impaired in the salivary glands, spleen and liver.[62] Additionally, a significant lower mortality rate in infected animals was observed compared to animals infected with wildtype virus.[62,68] Currently, not much is known about US27. As mentioned before, no ligands have been identified for this receptor. However, it has been shown that US27 is present in infected cells and enveloped virus particles, suggesting that this receptor could initiate or contribute to signal transduction events during the early stages of HCMV infection.[60,76,77]

US28 is currently the best characterized GPCR encoded by HCMV. The exact role of US28 has not been elucidated yet, but many important roles during viral infection have been attributed to this receptor (Figure 3).[70] The 30% amino acid sequence homology with the CC chemokine receptor CCR1[13] suggests that HCMV exploits chemokine signaling pathways to interfere with the host immune system through chemokine mimicry.[34]

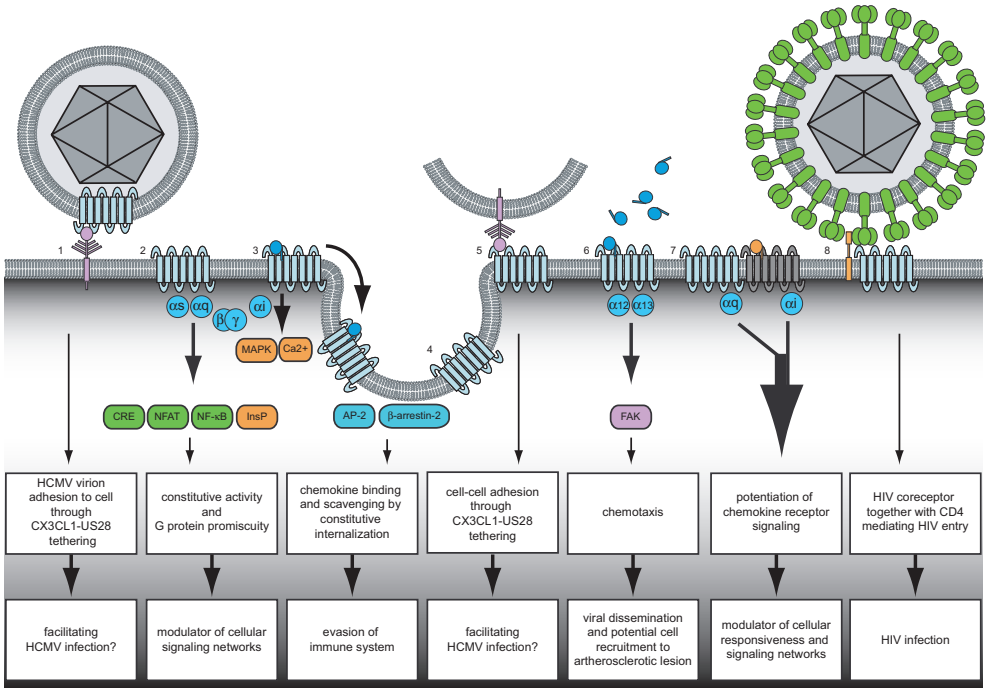


Figure 3. Schematic presentation of the putative roles of the HCMV-encoded receptor US28. Abbreviations: CRE, cAMP response element; FAK, focal adhesion kinase; InsP, inositol phosphate; NFAT, nuclear factor of activated T cells; NF-kB, nuclear factor kB. Adapted from Vischer et al.[70]

In contrast to human chemokine receptors, of which most receptors only bind one class of chemokines, US28 shows a large spectrum chemokine-binding profile and can bind a number of inflammatory CC chemokines, such as CCL2, CCL3, CCL4, CCL5, and CCL7, as well as the CX3C chemokine CX3CL1 with high affinity.[12-14,78] Due to its promiscuous chemokine-binding profile it is suggested that US28 could act as a chemokine scavenger by sequestering CC chemokines from the extracellular environment and thereby helps to subvert the host immune system.[67,79] This could be a way of the virus to elude immune surveillance due to the important role of chemokines in the regulation of the immune response. In addition, it has been shown that HCMV-infected fibroblasts are capable of depleting endogenous CCL2 and CCL5 from media in a US28-dependent manner, emphasizing that the chemokine binding properties of US28 could be a novel antichemokine mechanism for immune evasion.[66,67]

US28 activates different signaling pathways, such as phospholipase C, cAMP-response element binding protein (CREB) and nuclear factor activated T cell (NFAT) in an agonist-independent manner in both transiently transfected COS-7 cells as well as in HCMV-infected cells US28.[76,80,81] Next to this, US28 constitutively activate NF-κB, a transcription factor that plays a critical role in the regulation of inducible genes in immune response and inflammatory events associated with, for example, atherosclerosis.[82] Though the biological relevance of constitutive activity of vGPCRs has not been elucidated yet, it is believed to play an important role in the pathogenesis of virus infection. This has been shown for the HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV) encoded GPCR ORF74. This receptor is a viral oncogene that constitutively activates multiple intracellular signaling pathways resulting in the production and secretion of vascular endothelial growth factor (VEGF), thereby causing cellular transformations that can develop into highly vascularized Kaposi's sarcoma-like lesions in transgenic mice.[83,84]

CC chemokines act as agonists on US28 by inducing an increase in intracellular calcium in both transfected and HCMV-infected cells and by activating mitogen-activated protein kinase (MAPK) and focal adhesion kinase (FAK).[41,85] In contrast, they do not modulate the constitutive signaling of phospholipase C,

NF-κB, or CREB, thereby acting as neutral antagonists on these signaling pathways.[80,86] The CX3C-chemokine CX3CL1 partially inhibits the constitutive signaling of phospholipase C and the transcription factor NF-κB, thereby acting as a partial inverse agonist.[80] For US28 it has been shown to undergo rapid and constitutive endocytosis, so only a fraction of US28 is present on the cell surface.[87] Interestingly, CX3CL1 is reported to act as (partial) agonist on a *C*-terminal truncated US28 receptor (US28Δ300), which has a reduced constitutive internalization resulting in an increase in the number of receptors on the cell surface. Apparently, on wild type US28, CX3CL1 decreases the US28 surface levels and consequently the US28-mediated constitutive signaling. Thus, it seems that the agonistic properties of CX3CL1 are concealed by the constitutive endocytosis of wild type US28.[87] CX3CL1 is a membrane-bound chemokine and binding of US28 to CX3CL1 is suggested to play a role in the cell to cell transfer of HCMV.[78] Moreover, it could be hypothesized that US28 plays a role in the fusion of the CMV envelope with target cells and CMV entry due to its property to enhance cell-cell fusion mediated by different viral proteins.[88]

Similar to other mammalian chemokine receptors, of which CCR5 and CXCR4 are the primary HIV-1 co-receptors in vivo, US28 can act as a co-receptor for HIV-1 entry into cells in vitro.[89] Interestingly, a potential symbiotic relationship between the two viruses has been suggested, because HIV-1 establishes the immunosuppression needed for the emergence of HCMV from latency and maximal HCVM replication, while HCMV facilitates HIV-1 infection through US28 and provides an additional mechanism for cell entry by HIV-1.[27]

Another feature that increases the interest of US28 as a drug target is induction of the migration of vascular smooth muscle cells upon binding with the chemokines CCL2 and CCL5. This could be exploited by HCMV to disseminate the virus through the human body.[90] Since migration of smooth muscle cells towards the vascular intima is a typical characteristic of the formation of atherosclerotic and restenotic lesions, this could also provide a possible connection between HCMV and the development of vascular diseases.

During infection with HCMV different growth factors and cytokines are upregulated resulting in enhanced cell survival, proliferation and angiogenesis.[91] Recent experiments demonstrated that expression of US28 in vitro induces a transformed and pro-angiogenic phenotype. Additionally, activation of pro-angiogenic signaling pathways was apparent in HCMV-infected cells and this could be partially attributed to US28. In vivo, expression of US28 in NIH-3T3 cells promotes tumorigenesis in mice, thereby acting as a viral oncogene.[92] As such, after HCMV infection US28 might act in a concerted manner with other HCMV-encoded proteins, which were previously linked to oncogenesis,[91] and enhance and/or promote tumorigenesis.

Currently, the exact role of US28 in the pathogenesis of HCMV infection has not been completely elucidated yet. Because US28 is only present in primate CMV, this species-specificity as well as ethical considerations hampers the possibility to study the role of this receptor during viral infection in vivo. Consequently, more research is needed to further prove a causative link between US28 and chronic diseases resulting from infection with HCMV.

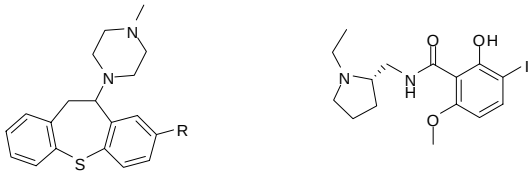
Small nonpeptidergic ligands acting on viral-encoded GPCRs

Because of the involvement of chemokines and chemokine receptors in the pathophysiology of numerous diseases, chemokine receptors were discovered as interesting new therapeutic targets. Chemokine receptors belong to Class A GPCRs, which are characterized by their high homology with rhodopsin. Other members of this family are the biogenic amine receptors, that have been successfully targeted by small-molecule antagonists and thus constitute a solid database of knowledge that should aid the design of novel antagonists for other GPCRs.[2] Drug development programmes were started in order to find small molecules acting on chemokine receptors and high-throughput-screenings resulted in several potent and selective antagonists currently entering Phase II or III clinical trials.

With the discovery of GPCRs encoded by viruses a new unexplored class of potential drug targets has emerged and selective small nonpeptidergic compounds that are able to inhibit these receptors can serve as new promising

therapeutics for innovative anti-viral intervention. Moreover, these small molecules can be used to study the relevance of constitutive signaling during viral infection. Currently, the HCMV encoded receptor US28 is the only viral-encoded GPCR for which small non-peptide molecules have been identified. Several classes of ligands that inhibit chemokine binding to US28 have been reported in the patent literature. Additionally, a series of inverse agonists acting on US28 were published recently by our group.[81]

In 2002, Chemocentryx Inc. disclosed that dissemination of CMV in a host could be inhibited by compounds that reversibly block chemokine binding to US28.[93,94] Among these reported ligands are a series of piperazinyldibenzothiepins, represented by the 5-hydroxytryptamine (5-HT) receptor antagonist methiothepin (**1**) and the D_2 dopamine/5-HT_2 antagonist octoclothepin (**2**) (Figure 4).



Methiothepin (**1**) R = SCH_3
Octoclothepin (**2**) R = Cl

S(-)-IBZM (**3**)

Figure 4. Chemical structures of the US28 ligands methiothepin, octoclothepin and S(-)-IBZM.

These tricyclic compounds have been shown to specifically inhibit [^{125}I]CX3CL1 binding to US28-expressing cells in a reversible manner with IC_{50} values of 0.3 μM and 0.7 μM, respectively.[94] In cytoplasmic calcium mobilization experiments in US28-expressing HEK293 cells, CX3CL1 acts as an agonist by inducing a rise in intracellular Ca^{2+}. Interestingly, both methiothepin and octoclothepin act as agonists in the same assay and are able to desensitize the subsequent Ca^{2+} mobilization by CX3CL1.

A family of benzamides, exemplified by S(-)-IBZM (**3**) in Figure 4, has been reported as ligands interacting with US28.[93] S(-)-IBZM is known as a D_2 dopamine receptor ligand, which can be radiolabeled due to the presence of a

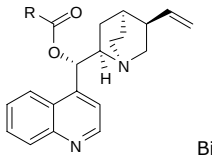
synthetically accessible iodide substituent in the structure. Radiolabeled **3** is used in the clinic to visualize D_2-dopamine receptors in vivo in the human brain by single photon emission tomography (SPECT).[95] As shown for methiothepin and octoclothepin, S(-)-IBZM is able to specifically displace [^{125}I]CX3CL1 binding from US28 with an IC_{50} value of 0.6 μM and to act as an agonist in the calcium mobilization assay. Due to the observed interaction with US28, it was claimed that [^{123}I]IBZM could be used for the in vivo detection, diagnosis and imaging of CMV infection in a host using single photon emission computed tomography (SPECT) (or positron emission tomografie (PET) if ^{18}F or another positron emitter is used). Yet, in view of the relatively low micromolar affinity of S(-)-IBZM on US28, the feasibility of this approach seems questionable.

Although in general, caution must be taken when trying to deduce structure-activity relationships (SAR) from patent literature, we will try to identify general trends or remarkable issues, e.g. selectivity, in the following subsections.

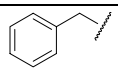
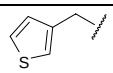
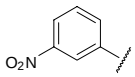
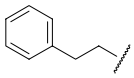
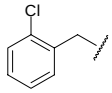
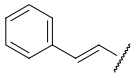
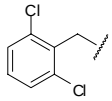
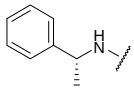
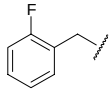
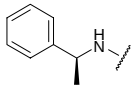
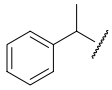
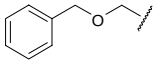
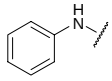
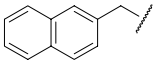
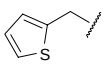
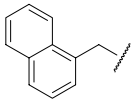
A large series of bicyclic compounds, represented by compounds **4-53**, were reported for the treatment or prevention of viral dissemination from CMV infection by inhibiting chemokine binding to US28.[96] Bicyclic structures A-D (Table 3-6) were reacted with a large series of acid chlorides and these derivatives were evaluated for their binding properties by their ability to inhibit [^{125}I]CX3CL1 binding to human US28-expressing cells and to rhesus CMV-infected rhesus dermal fibroblasts. Interestingly, a striking species-selectivity between human US28 and rhesus CMV was demonstrated. Several tested ligands exhibited IC_{50} values lower than 1 μM on human US28 or rhesus CMV, but only compound **49** showed a binding affinity below 1 μM in both systems. Recently, species selectivity has been shown for CCR1 antagonist as well and this could be inconvenient if these molecules are tested in animal models of disease.[97]

All compounds based on bicyclic structure A are shown in Table 3. In detail, the unsubstituted analogue **4** and the different substituted compounds **5**-**8** have an affinity < 1 μM on human US28, while none of these compounds show a similar affinity on rhesus CMV. The introduction of a methyl group in **9** or the nitrogen atom in **10** causes a drop in affinity for both receptors compared to unsubstituted compound **4**. The thiophene groups in **11** and **12** are allowed in the structure and both compounds have an affinity below 1 μM on hUS28, but this substitution results in a significant decrease on rhesus CMV.

Table 3. Chemical structures and pharmacological properties of compounds **4**-**19** for human US28 and rhesus CMV.



Bicyclic structure **A**

no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]	no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]
4		< 1	1 - 10	**12**		< 1	10 - 40
5		< 1	10 - 40	**13**		1 - 10	10 - 40
6		< 1	1 - 10	**14**		1 - 10	10 - 40
7		< 1	1 - 10	**15**		10 - 40	10 - 40
8		< 1	10 - 40	**16**		10 - 40	10 - 40
9		1 - 10	10 - 40	**17**		1 - 10	1 - 10
10		1 - 10	10 - 40	**18**		< 1	1 - 10
11		< 1	10 - 40	**19**		1 - 10	1 - 10

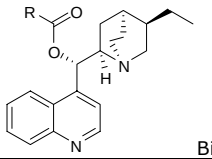
[a] Displacement of [^{125}I]CX3CL1 from human US28-expressing cells. [b] Displacement of [^{125}I]CX3CL1 from rhesus CMV-infected rhesus dermal fibroblasts.

The introduction of an additional carbon atom in the structure of **13** or the unsaturated system of **14** does not positively influence the affinity on hUS28 or rhesus CMV, while the substituted amine groups of the enantiomerically pure compounds **15** and **16** cause a decrease in affinity compared to **4**. The presence of the ether linkage between the phenyl ring and bicyclic structure A (**17**) results in an affinity between 1 and 10 μM on both hUS28 as rhesus CMV. The same is seen for the introduction of a 1-naphtylgroup (**19**), while the 2-naphtyl group in compound **18** does not influence the affinity on rhesus CMV, but causes an increase in binding affinity on hUS28 to a value below 1 μM.

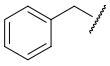
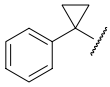
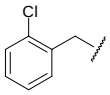
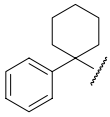
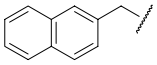
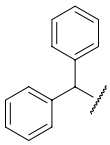
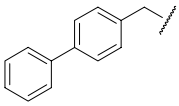
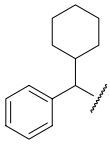
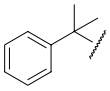
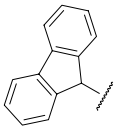
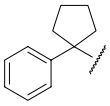
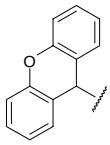
The only difference between the bicyclic structures A (Table 3) and B (Table 4) is the presence of a double or single bond in the carbon chain attached to the 3-position of the piperidine ring. The influence of this small structural change is not significant. Both the introduction of a benzyl group (**20**), a 2-chloro benzyl group (**21**) or a 2-naphtyl group (**22**) results in similar binding affinities as their analogues in Table 3. Moreover, 4-phenyl substitution as in compound **23** was allowed to maintain affinity on both human as rhesus CMV. The two methyl groups in compound **24** do not positively influence the affinity on hUS28 (compare to compound **20**), and this structural change causes a drop in affinity on US28 expressed by rhesus CMV.

An interesting series with different cycloalkyl groups attached to the benzylic carbon atom was synthesized and tested. On human US28, there are no differences in binding affinity between the cyclopentyl group in **25**, the cyclopropyl group in **26** or the cyclohexyl group in **27**, while the last substituent is preferred on rhesus CMV. Replacement of one of the phenyl rings in **28** by a cyclohexyl ring as in **29** causes a small decrease in affinity on both hUS28 as rhesus CMV, while the tricyclic systems in compounds **30** and **31** result in binding affinities between 1 and 10 μM on both receptors.

Table 4. Chemical structures and pharmacological properties of compounds **20**-**31** for human US28 and rhesus CMV.



Bicyclic structure **B**

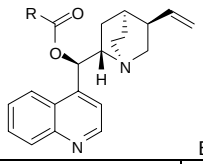
no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]	no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]
20		< 1	10 - 40	**26**		1 - 10	10 - 40
21		< 1	1 - 10	**27**		1 - 10	1 - 10
22		< 1	1 - 10	**28**		< 1	1 - 10
23		< 1	1 - 10	**29**		1-10	10 - 40
24		1 - 10	10 - 40	**30**		1-10	1 - 10
25		1 - 10	10 - 40	**31**		1-10	1 - 10

[a] Displacement of [^{125}I]CX3CL1 from human US28-expressing cells. [b] Displacement of [^{125}I]CX3CL1 from rhesus CMV-infected rhesus dermal fibroblasts.

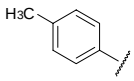
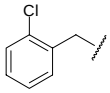
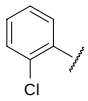
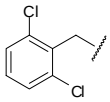
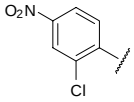
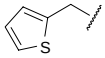
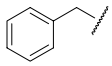
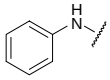
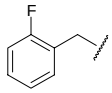
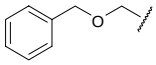
Compounds **32**-**41** are based on bicyclic structure C (Table 5). Changing the substitution in the phenyl ring from a 4-methyl group to 2-chloro or 2-chloro-4-nitro substitution (compare compounds **33** and **34** with **32**) causes a small drop in affinity on hUS28, while this structural change increases the IC_{50} value on rhesus CMV from a value between 1-10 μM to a value below 1 μM. In contrast to ligands **4**-**8** in Table 3, which all have affinities below 1 μM, the compounds with a benzyl group in **35** or the substituted benzyl groups in **36**-**38** have affinities

on hUS28 between 1-10 μM. On rhesus CMV, the unsubstituted benzyl group in compound **35** or the 2-chloro and 2,2-dichloro substituted analogues **37** and **38** are preferred above the 2-fluoro substituted benzyl group in compound **36**. Introduction of the nitrogen linker in **40** or the ether linker as in compound **41** does not increase the affinities on human or rhesus CMV.

Table 5. Chemical structures and pharmacological properties of compounds **32**-**41** for human US28 and rhesus CMV.



Bicyclic structure **C**

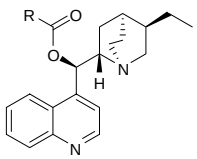
no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]	no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]
32		< 1	1 - 10	**37**		1 - 10	< 1
33		1 - 10	< 1	**38**		1 - 10	< 1
34		1 - 10	< 1	**39**		< 1	1 - 10
35		1 - 10	< 1	**40**		1 - 10	10 - 40
36		1 - 10	1 - 10	**41**		1 - 10	1 - 10

[a] Displacement of [^{125}I]CX3CL1 from human US28-expressing cells. [b] Displacement of [^{125}I]CX3CL1 from rhesus CMV-infected rhesus dermal fibroblasts.

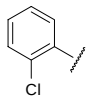
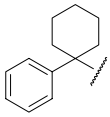
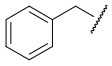
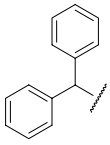
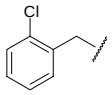
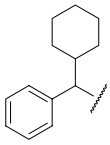
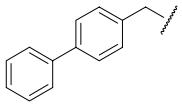
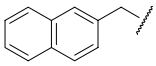
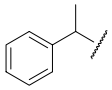
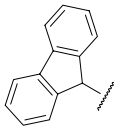
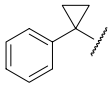
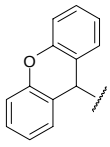
Interestingly, small changes in affinity are seen if the double bond in bicyclic structure C is replaced by a single C-C bond as in scaffold D (Table 5 and 6). Analogue **42** has a comparable affinity on hUS28 as compound **33**, but the presence of the single bond in bicyclic structure D causes a small decrease in binding affinity on rhesus CMV. As shown for analogues **4** and **20**, the benzyl group in compound **43** results in a binding affinity lower than 1 μM on hUS28. The introduction of a 2-chloro substituent (**44**), a 4-phenyl group (**45**) or a

methyl group at the benzylic position (**46**) are allowed to maintain for an affinity between 1-10 μM on human US28 and rhesus CMV.

Table 6. Chemical structures and pharmacological properties of compounds **42**-**53** for human US28 and rhesus CMV.



Bicyclic structure **D**

no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]	no.	R	hUS28 IC_{50} (μM)[a]	rhesus CMV IC_{50} (μM)[b]
42		1 - 10	1 - 10	**48**		1 - 10	< 1
43		< 1	1 - 10	**49**		< 1	< 1
44		1-10	1 - 10	**50**		1 - 10	< 1
45		1 - 10	1 - 10	**51**		1 - 10	< 1
46		1 - 10	1 - 10	**52**		1 - 10	< 1
47		< 1	1 - 10	**53**		1 - 10	< 1

[a] Displacement of [^{125}I]CX3CL1 from human US28-expressing cells. [b] Displacement of [^{125}I]CX3CL1 from rhesus CMV-infected rhesus dermal fibroblasts.

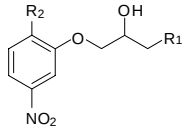
If the results of compounds **25** and **27** (bicyclic structure B) are compared with the binding affinities of compounds **47** and **48**, it is interesting to notice that in bicyclic structure D the introduction of a cyclopropyl group (**47**) results in an

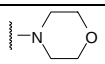
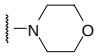
affinity lower than 1μM on hUS28, while the cyclohexyl group of **48** is preferred on rhesus CMV.

As mentioned before, compound **49** is the only compound that has an affinity lower than 1 μM on both human US28 as rhesus CMV. Replacement of one of the phenyl rings of **49** by a cyclohexyl group as in **50** results in a small drop of affinity on only hUS28, and this is also seen for compound **51**. The same tricyclic systems as in compounds **30** and **31** (bicyclic structure B) were introduced on bicyclic structure D to afford compounds **52** and **53**. Interestingly, in bicyclic structure B these substitutions result in affinities between 1-10 μM on both receptors, while in bicyclic structure D the tricyclic substitutions show a slight preference for US28 encoded by rhesus CMV.

The last group of compounds disclosed by Chemocentryx Inc. as inhibitors of chemokine binding to US28 is exemplified by compounds **54**-**65** (Table 7).[98] The activities of these arylamines were determined by their ability to inhibit [^{125}I]CX3CL1 binding to US28-expressing cells. As for the bicyclic compounds, the most active compounds were claimed to have IC_{50} values lower than 1 μM, but specific IC_{50} values and functional data were not reported. A more detailed look into the SAR of these arylamines reveals that two small alkyl groups on the nitrogen atom attached to the phenoxy linker, like shown for **54**-**55**, is preferred. Monosubstitution of the nitrogen atom as in compound **56** or the introduction of a more bulky *t*-butyl group as in **57** makes the affinity drop slightly. Incorporation of the nitrogen atom into a ring system, e.g. in a pyrrolidine (**58**) or piperidine ring (**59**), makes the binding affinity drop slightly as well, while introduction of a morpholine ring in compound **60** causes a more significant drop in IC_{50} value. Interestingly, the NO_2 group of compound **54** seems to be essential in the structure, because replacement by a CF_3 group results in a decrease in binding affinity to 1–10 μM, and introduction of a NH_2 or a $COOCH_3$ moiety even causes the IC_{50} values to drop between 10-40 μM (structures not shown).

Table 7. Chemical structures and pharmacological properties of compounds **54**-**65** for the HCMV-encoded receptor US28.



no.	R_1	R_2	IC_{50} (μM)[a]	no.	R_1	R_2	IC_{50} (μM)[a]
54			< 1	**60**			10 – 40
55			< 1	**61**			< 1
56			1 - 10	**62**			10 – 40
57			1 - 10	**63**			< 1
58			1 - 10	**64**			10 - 40
59			1 - 10	**65**			10 – 40

[a] Displacement of [^{125}I]CX3CL1.

Introduction of a methyl substituent into the ring system attached to the phenyl ring (**61**) is allowed, but replacement of the pyrrolidine ring into a morpholine ring as in compound **62** results in a drop in affinity. An isopropyl group attached to the aniline nitrogen atom in **63** results in an IC_{50} value below 1 μM, while two methyl substituents in compound **64** make the affinity drop to a value between 10 and 40 μM. Removal of any substituent in that position of the molecule in **65** shows the same drop in IC_{50} value.

Previously, VUF2274 (**66**) was identified as the first small non peptide molecule acting as an inverse agonist on US28 (Table 8).[81] This molecule has been previously reported as an antagonist on the human chemokine receptor CCR1[99] and was screened on US28 because of the sequence homology of this viral receptor with CCR1 (33% identity). Compound **66** has been shown to displace [^{125}I]CCL5 binding to US28-expressing COS-7 cells with an IC_{50} value of 9.3 μM. In previous studies it was shown that in transiently transfected COS-7 cells as well as in HCMV-infected cells US28 constitutively activates phospholipase C and the transcription factor NF-κB in an agonist-independent manner.[80,81]

Constitutive signaling of US28 could be completely blocked by **66** with an EC_{50} value of 3.2 μM. VUF2274 does not only dose-dependently inhibit the US28-mediated constitutive activation of PLC in both transiently transfected cells and HCMV-infected fibroblasts, but also inhibits the US28-mediated HIV entry in cells cotransfected with CD4.[81]

To investigate the binding sites of US28 an *in silico* model was generated based on homology with bovine rhodopsin (Figure 5).[81] A glutamic acid residue in TM-7 (Glu^{277}) was identified as an important amino acid for binding of **66** to the receptor, because mutation into glutamine to eliminate the charge but to maintain the hydrogen bonding potential of the side chain, and into alanine to eliminate both the charge as well as the hydrogen bonding potential, significantly reduced the binding affinity of **66**. The importance of a glutamate residue in TM-7 for binding and action of nonpeptidergic chemokine antagonists has been shown for the human CCR1, CCR2 and CCR5 chemokine receptors as well.[100,101] Moreover, it has been suggested for human chemokine receptors in general that this residue serves as an important anchor point for the positively charged nitrogen atom, which is frequently found in small molecule inhibitors acting on these receptors.[102]

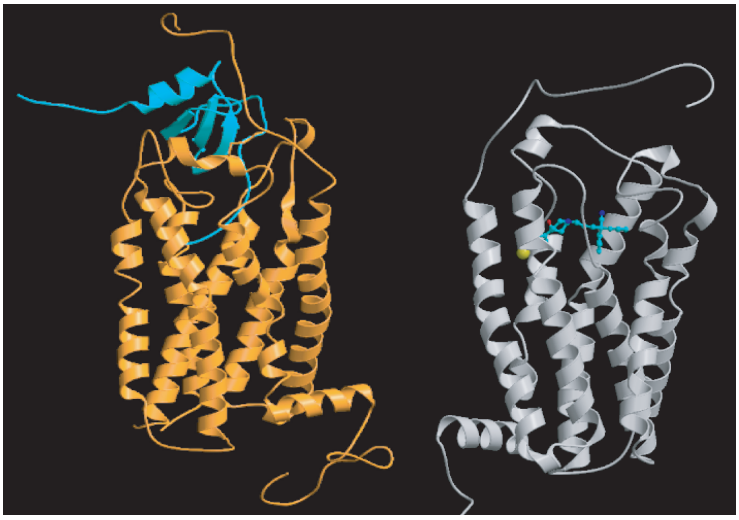
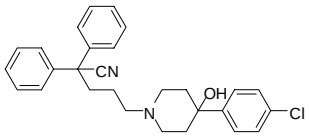
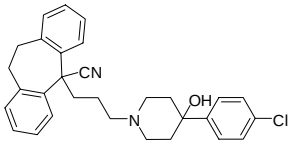
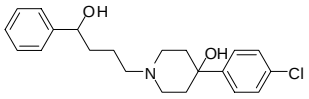
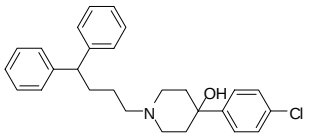
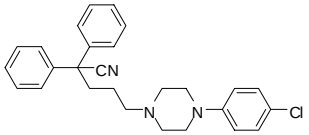
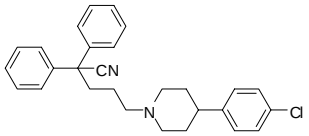
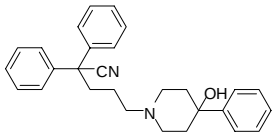


Figure 5. *In silico* model of US28. Left: Proposed binding site of CCL5 (cyan) with US28. Right: Proposed binding site of **66** within the transmembrane helices of US28.

As expected, it was shown that **66** and the relatively large chemokine CCL5 do not share the same binding site on US28. Firstly, Glu^{277} was not important for CCL5 binding to the receptor. Secondly, mutation of the *N*-terminus of US28 (Δ(2-22)-US28) resulted in a complete loss of binding affinity of all the tested chemokines, but the constitutive signaling of this mutant receptor could still be inhibited by **66** with a potency similar to that of wild type US28. Thus, compound **66** and CCL5 do not share the same binding site on US28, but the small molecule acts as an allosteric modulator and displacement of CCL5 binding results from a conformational change induced by **66**.[81]

Table 8. Chemical structures and pharmacological properties of compounds **66**-**72** for the HCMV-encoded receptor US28.

no.	Code	Structure	IC_{50} (μM)[a]	EC_{50} (μM)[b]
66	2274		8.4	3.5
67	5713		9.6	5.3
68	(±)-5715		n.a	n.a.
69	5667		5.9	4.2
70	5658		n.a	n.a.
71	5662		10.5	14
72	5660		35	29

[a] Displacement of [^{125}I]CCL5. [b] Inhibition of [^{3}H]inositol phosphate production. n.a.= not active.

A limited series of analogues of **66** was synthesized to investigate some preliminary structure-activity relationships for inverse agonism on US28 (Table 7).[81] Some flexibility was tolerated in the diphenylacetonitrile group, because insertion of an ethylene group as in VUF5713 (**67**) or a thiomethylene group (structure not shown) was tolerated and did not influence the affinity and efficacy of the compounds. In contrast, replacement of one of the two phenyl rings by a more hydrophilic hydroxy group as in compound **68** resulted in a complete loss of activity, suggesting that a bulky lypophilic moiety is of importance. The nitrile group was not essential in the structure, because its removal in VUF5667 (**69**) did not affect the affinity and efficacy of the compound. Interestingly, replacement of the piperidine ring of **66** into a piperazine ring in VUF5658 (**70**) resulted in a complete loss of activity. Finally, removal of the hydroxy group attached to the piperidine ring in VUF5662 (**71**) or the chloride substituent in VUF5660 (**72**) resulted in a 5-10 fold loss of affinity and efficacy.

At present, no low molecular weight ligands are known to act on other vGPCRs, but this could be only a matter of time. Previously, it was shown that ORF74, a constitutively active GPCR encoded by HHV-8, was susceptible to non-peptide inverse agonists by inhibition of the constitutive signaling of the viral receptor by a Zn^{2+} ion.[29] To this end, a silent metal ion site was constructed by His-substitution of Arg^{208} and Arg^{212} and the signaling of this ORF74 mutant receptor was blocked by Zn^{2+}, which acted as an inverse agonist with an EC_{50} around 1 μM. This was suggested to be a proof of concept that it is possible to identify small nonpeptidergic inverse agonists targeted towards the extracellular part of this viral-encoded GPCR.

In summary, the search for small ligands acting on vGPCRs is still an unexplored but fascinating research field. The identification of these nonpeptidergic molecules is essential to investigate the role of vGPCRs in the pathogenesis of viral infection and these molecules can be considered as promising therapeutics for clinical anti-viral intervention.

Aim of this thesis

As outlined above, HCMV infection has been linked to numerous pathological disorders, such as vascular diseases, transplant rejection, several inflammatory and autoimmune diseases, as well as cancer. Furthermore, HIV patients co-infected with HCMV have an increased risk of rapid disease progression. US28 is one of at least four chemokine receptors encoded by HCMV and this receptor is suggested to play a significant role in virus-associated diseases. US28 binds several inflammatory chemokines and displays a high level of constitutive (ligand-independent) signaling by activating various signaling pathways. Aberrant activation of signaling pathways by GPCRs has been recognized as a potential cause of numerous human pathologies. Inverse agonists are able to inhibit this constitutive activation. The identification of such molecules for viral-encoded GPCRs can be an important tool to investigate the significance of constitutive activity and the role of these receptors in viral infection.

The aim of this thesis is to elucidate the first structure-activity relationships for inverse agonism on the viral-encoded receptor US28 by the design, synthesis and pharmacological evaluation of novel nonpeptidergic ligands. These insights will result in a better understanding of the structural requirements important for inverse agonism and ultimately lead to compounds with an improved activity and selectivity profile on US28. Moreover, these new molecules may serve as important tools to investigate the role of US28-mediated constitutive activity during viral infection.

Chapter 2 and 3 describe the synthesis and pharmacological characterization of an extensive series of analogues of compound **1**, identified in our lab as the first nonpeptidergic ligand acting on US28. This comprehensive study describes the synthesis of several series of compounds and the very first SAR of nonpeptidergic ligands acting on a viral GPCR (US28).
Chapter 4 shows an interesting approach using molecular design techniques to identify novel ligands for US28. The design of a focused virtual library around 4-(4-chlorophenyl)piperidin-4-ol will be shown and how drug-like descriptors were applied to compose a diverse subset of 50 compounds. These molecules were

synthesized using parallel chemistry approaches and subsequently evaluated pharmacologically on US28.

Chapter 5 discloses the results of our quest for novel chemotypes interacting with US28 by screening a selection of compounds from our own in-house compound collection. In this study, several interesting ligands have been found, of which some are selected as new starting points for further lead optimization programs. Interestingly, two of these compounds were classified as a neutral antagonist, providing us with a new pharmacological tool.

Chapter 6 shows the synthesis of a hybrid compound that contains important structural features of both compound **1** and one of the neutral antagonists. This hybrid molecule led to the discovery of a molecular determinant that leads to neutral antagonism or inverse agonism. These compounds give us a better understanding of the structural requirements necessary for inverse agonism and neutral antagonism and the possibility to modulate the functional activity of US28.

Chapter 7 describes the hit to lead optimization of one of the novel inverse agonists that was identified by our in-house database screening. We describe some interesting new structure-activity relationships of this completely novel class of inverse agonists acting on US28.

References

1. Gurrath, M. Peptide-binding G protein-coupled receptors: new opportunities for drug design. *Curr. Med. Chem.* **2001**, *8*, 1605-1648.
2. Onuffer, J. J.; Horuk, R. Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol. Sci.* **2002**, *23*, 459-467.
3. Müller, A.; Homey, B.; Soto, H.;Ge, N.;Catron, D.; Buchanan, M. E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S. N.; Barrerak, J. L.; Mohark, A.; Verástegui, E.; Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature* **2001**, *410*, 50-56.
4. Fernandez, E. J.; Lolis, E.; Structure, function, and inhibition of chemokines. *Annu. Rev. Pharmacol. Toxicol.* **2002**, *42*, 469-499.
5. Mackay, C. R. Chemokines: immunology's high impact factors. *Nat. Immunol.* **2001**, *2*, 95–101.
6. Murphy, P. M.; Baggiolini, M.; Charo, I. F.; Herbert, C. A.; Horuk, R.; Matsushima, K.; Miller, L. H.; Oppenheim, J. J.; Power, C. A. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev.* **2002**, *52*, 145-176.
7. Charo, I. F.; Ransohoff, R. M. The many roles of chemokines and chemokine receptors in inflammation. *N. Engl. J. Med.* **2006**, *354*, 610-621.
8. Rot, A.; von Andrian, U. H. Chemokines in innate and adaptive hist defense: basic chemokinese grammar for immune cells. *Annu. Rev. Immunol.* **2004**, *22*, 891–928.
9. Proudfoot, A. E. I.; Power, C. A.; Wells, T. N. C. The strategy of blocking the chemokine system to combat disease. *Immunol. Rev.* **2000**, *177*, 246–256.
10. Mantovani, A. The chemokine system: redundancy for robust outputs. *Immunol. Today* **1999**, *20*, 254-257.
11. Horuk, R.; Chitnis, C. E.; Darbonne, W. C.; Colby, T. J.; Rybicki, A.; Hadley, T. J.; Miller, L. H. A receptor for the malarial parasite *Plasmodium* vivax: The erythrocyte chemokine receptor. *Science* **1993**, *261*, 1182–1184.

12. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
13. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
14. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.
15. Nibbs R. J.; Wylie, S. M.; Yang, J.; Landau, N. R.; Graham, G. J. Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6. *J. Biol. Chem.* **1997**, *272*, 32078–32083.
16. Burns, J. M.; Summers, B. C.; Wang, Y.; Melikian, A.;Berahovich, R.; Miao, Z.; Penfold, M. E. T.; Sunshine, M. J.; Littman, D. R.; Kuo, C. J.; Wei, K.; McMaster, B. E.; Wright, K.; Howard, M. C.; Schall, T. J. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J. Exp. Med.* **2006**, 203, 2201-2213.
17. Baggiolini, M.; Dewald, B.; Moser, B. Human chemokines: an update. *Annu. Rev. Immunol.* **1997**, *15*, 675–705.
18. Berger E. A. HIV entry and tropism: the chemokine receptor connection. *Aids* **1997**, *11*, S3–S16.
19. Dalgleish A. G.; Beverley P. C.; Clapham P. R.; Crawford D. H.; Greaves M. F.; Weiss R. A.; The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **1984**, *312*, 763–767.
20. Simmons, G.; Reeves, J. D.; Hibbitts, S.; Stine, J. T.; Gray, P. W.; Proudfoot, A. E. I.; Clapham, P. R. Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol. Rev.* **2000**, *177*, 112-126.
21. Stephens, J. C.; Reich, D. E.; Goldstein, D. B.; Shin, H. D.; Smith, M. W.; Carrington, M.; Winkler, C.; Huttley, G. A.; Allikmets, R.; Schrim, L.; Gerrard, B.; Malasky, M.; Ramos, M. D.; Morlot, S.; Tzetis, M.; Oddoux, C.; diGiovine, F. S.; Nasioulas, G.; Chandler, D.; Aseev, M.; Hanson, M.; Kalaydjieva, L.; Glavac, D.; Gasparini, P.; Dean, M. Dating the origin of the CCR5-D32 AIDS-resistance allele by the coalescence of haplotypes. *Am. J. Hum. Genet.* **1998**, *62*, 1507–1515.
22. De Silva, E.; Stumpf, M. P. H. HIV and the CCR5-Δ32 resistance allele. *FEMS Microbiol. Lett.* **2004**, *241*, 1–12.
23. Benkirane, M.; Jin, D.-Y.; Chun, R. F.; Koupi, R. A.; Jeang, K.-T. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by CCR5Δ32. *J. Biol. Chem.* **1997**, *272*, 30603–30606.
24. Wells, T. N. C.; Power, C. A.; Shaw, J. P.; Proudfoot, A. E. I. Chemokine blockers–therapeutics in the making? *Trends Pharmacol. Sci.* **2006**, *27*, 41-47.
25. Rosenkilde, M. M.; Waldhoer, M.; Lüttichau, H. R.; Schwartz, T.W. Virally encoded 7TM receptors. *Oncogene* **2001**, *20*, 1582-1593.
26. Rosenkilde, M. M. Virus-encoded chemokine receptors - putative novel antiviral drug targets. *Neuropharmacology* **2005**, *48*, 1-13.
27. Pease, J. E.; Murphy, P. M. Microbial corruption of the chemokine system: an expanding paradigm. *Semin. Immunol.* **1998**, 10, 169-178.
28. Luttichau, H. R.; Stine, J.; Boesen, T. P.; Johnsen, A. H.; Chantry, D.; Gerstoft. J.; Schwartz, T. W. A highly selective CC chemokine receptor (CCR)8 antagonist encoded by the poxvirus molluscum contagiosum. *J. Exp. Med.* **2000**, *191*, 171-180.
29. Rosenkilde, M. M.; Kledal, T. N.; Bräuner-Osborne, H.; Schwartz, T. W. Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product ORF-74. *J. Biol. Chem.* **1999**, *274*, 956–961.
30. Webb, L. M. C.; Alcami, A. Virally encoded chemokine binding proteins. *Mini Rev. Med. Chem.* **2005**, *5*, 833-848.
31. Lalani, A. S.; Graham, K.; Mossman, K.; Rajarathnam, K.; Clark-Lewis, I.; Kelvin, D.; McFadden, G. The purified myxoma virus gamma interferon receptor homolog M-T7 interacts with the heparin-binding domains of chemokines. *J. Virol.* **1997**, *71*, 4356–4363.
32. Alcami, A. Interaction of viral chemokine inhibitors with chemokines. *Methods Mol. Biol.* **2004**, *239*, 167–180.
33. van Berkel, V.; Preiter, K.; Virgin, H. W. IV; Speck, S. H.; Identification and initial characterization of the murine gammaherpesvirus 68 gene M3, encoding an abundantly secreted protein. *J. Virol.* **1999**, *73*, 4524–4529.
34. Murphy, P. M. Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat. Immunol.* **2001**, *2*, 116-122.
35. Ray, N.; Doms, R. W. HIV-1 coreceptors and their inhibitors. *Curr. Top. Microbiol. Immunol.* **2006**, *303*, 97-120.

36. Smit, M. J.; Vink, C.; Verzijl, D.; Casarosa, P.; Bruggeman, C. A.; Leurs, R. Virally encoded G protein-coupled receptors: targets for potentially innovative anti-viral drug development. *Curr. Drug Targets* **2003**, *4*, 431-441.
37. Vischer, H. F.; Vink, C.; Smit, M.J. A viral conspiracy: hijacking the chemokine system through virally encoded pirated chemokine receptors. *Curr. Top. Microbiol. Immunol.* **2006**, *303*, 121-154.
38. Proudfoot, A. E. Chemokine receptors: multifaceted therapeutic targets. *Nat. Rev. Immunol.* **2002**, *2*, 106-115.
39. Britt, W. J. Vaccines against human cytomegalovirus: time to test. *Trends Microbiol.* **1996**, *4*, 34-38.
40. Landolfo, S.; Gariglio, M.; Gribaudo, G.; Lembo, D. The human cytomegalovirus. *Pharmacol. Ther.* **2003**, *98*, 269–297.
41. Streblow, D. N.; Vomaske, J.; Smith, P.; Melnychuk, R.; Hall, L.; Pancheva, D.; Smit, M.; Casarosa, P.; Schlaepfer, D. D.; Nelson, J. A. Human cytomegalovirus chemokine receptor US28-induced smooth muscle cell migration is mediated by focal adhesion kinase and Src. *J. Biol. Chem.* **2003**, *278*, 50456–50465.
42. Pass, R. F.; Epidemiology and transmission of cytomegalovirus. *J. Infect. Dis.* **1985**, *152*, 243–248.
43. Sohn, Y. M.; Oh, M. K.; Balcarek, K. B.; Cloud, G. A.; Pass, R. F. Cytomegalovirus infection in sexually active adolescents. *J. Infect. Dis.* **1991**, *163*, 460–463.
44. Schleiss, M. Progress in cytomegalovirus vaccine development. *Herpes* **2005**, *12*, 66-75.
45. Jarvis, M. A.;Nelson, J. A. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Curr. Opin. Microbiol.* **2002**, *5*, 403-407.
46. Gandhi, M. K; Khanna, R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect. Dis.* **2004**, *4*, 725-738.
47. Demmler, G. J. Congenital cytomegalovirus infection and disease. *Adv. Pediatr. Infect. Dis.* **1996**, *11*, 135–162.
48. Melnick, J. L.; Hu, C.; Burek, J.; Adam, E.; DeBakey, M. E. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* **1994**, *42*, 170-174.
49. Valantine, H. A. The role of viruses in cardiac allograft vasculopathy. *Am. J. Transplant.* **2004**, *4*, 169-177.
50. Zhou, Y. F.; Leon, M. B.; Waclawiw, M. A.; Popma, J. J.; Yu, Z. X.; Finkel, T.; Epstein, S. E. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **1996**, *335*, 624-630.
51. Stassen, F. R.; Vega-Cordova, X.; Vliegen, I.; Bruggeman, C. A. Immune activation following cytomegalovirus infection: more important than direct viral effects in cardiovascular disease? *J. Clin. Virol.* **2006**, *35*, 349–353.
52. Soderberg-Naucler, C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J. Intern. Med.* **2006**, *259*, 219-246.
53. Harkins, L.,; Volk, A. L.; Samanta, M.; Mikolaenko, I., Britt, W. J.; Bland, K. I.; Cobbs, C. S. Specific localisation of human cytomegalovirus nucleic acids and proteins in human colorectal cancer. *Lancet* **2002**, *360*, 1557–1563.
54. Cobbs, C. S.; Harkins, L.; Samanta, M.; Gillespie, G. Y.; Bharara, S.; King, P. H.; Nabors, L. B.; Cobbs, C. G.; Britt, W. J. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res.* **2002**, *62*, 3347–3350.
55. Razonable, R. R.; Paya, C. V. β-Herpesviruses in transplantation. *Rev. Med. Microbiol.* **2002**, *13*, 163–176.
56. Deayton, J. R.; Sabin, C. A.; Johnson, M. A.; Emery, V. C.; Wilson, P.; Griffiths, P. D. Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. *Lancet*, **2004**, *363*, 2116–2121.
57. Chee, M. S.; Satchwell, S. C.; Preddie, E.; Weston, K. M.; Barrel, B. G. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature* **1990**, *344*, 774-777.
58. Rigoutsos, I.; Novotny, J.; Huynh, T.; Chin-Bow, S. T.; Parida, L.; Platt, D.; Coleman, D.; Shenk, T. In silico pattern-based analysis of the human cytomegalovirus genome. *J. Virol.* **2003**, *77*, 4326-4344.
59. Lesniewski, M.; Das, S.; Skomorovska-Prokvolit, Y.; Wang, F.-Z.; Pellett, P. E. Primate cytomegalovirus US12 gene family: a distinct and diverse clade of seven-transmembrane proteins. *Virology* **2006**, *354*, 286-298.
60. Margulies, B. J.; Browne, H.; Gibson, W. Identification of the human cytomegalovirus G-protein-coupled receptor homologue encoded by UL33 in infected cells and enveloped virus particles. *Virology* **1996**, *225*, 111–125.
61. Fraile-Ramos, A.; Kledal, T. N.; Pelchen-Matthews, A.;Bowers, K.; Schwartz, T. W.; Marsh, M. The human cytomegalovirus US28 protein is located in endocytic vesicles and undergoes constitutive endocytosis and recycling. *Mol. Biol. Cell* **2001**, *12*, 1737-1749.

62. Oliveira, S. A.; Shenk, T. E. Murine cytomegalovirus M78 protein, a G protein-coupled receptor homologue, is a constituent of the virion and facilitates accumulation of immediate early viral mRNA. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 3237–3242.
63. Fraile-Ramos, A.; Pelchen-Matthews, A.; Kledal, T. N.; Brown, H.; Schwartz, T. W.; Marsh, M. Localization of HCMV UL33 and US27 in endocytic compartments and viral membranes. *Traffic* **2002**, *3*, 218-232.
64. Penfold, M. E. T.; Schmidt, T. L.; Dairaghi, D. J.; Barry, P. A.; Schall, T. J. Characterization of the rhesus cytomegalovirus US28 locus. *J. Virol.* **2003**, *77*, 10404–10413.
65. Davis-Poynter, N. J.; Lynch, D. M.; Vally, H.; Shellam, G. R.; Rawlinson, W. D.; Barell, B. G.; Farrell, H. E. Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. *J. Virol.*, **1997**, *71*, 1521–1529.
66. Bodaghi, B.; Jones, T. R.; Zipeto, D.; Vita, C.; Sun, L.; Laurent, L.; Arenzana-Seisdedos, F.; Virelizier, J. L.; Michelson, S. chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. *J. Exp. Med.* **1998**, *188*, 855-866.
67. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
68. Beisser, P. S.; Grauls, G.; Bruggeman, C. A.; Vink, C. Deletion of the R78 G protein-coupled receptor gene from rat cytomegalovirus results in an attenuated, syncytium-inducing mutant strain. *J. Virol.* **1999**, *73*, 7218–7230.
69. Michel, D.; Milotic, I.; Wagner, M.; Vaida, B.; Holl, J.; Ansorge, R.; Mertens, T. The human cytomegalovirus UL78 gene is highly conserved among clinical isolates, but is dispensable for replication in fibroblasts and a renal artery organ-culture system. *J. Gen. Virol.* **2005**, *86*, 297-306.
70. Vischer, H. F.; Leurs, R.; Smit, M. J. HCMV-encoded G-protein-coupled receptors as constitutively active modulators of cellular signaling networks. *Trends Pharmacol. Sci.* **2006**, *27*, 56-63.
71. Gruijthuijsen, Y. K.; Casarosa, P.; Kaptein, S. J. F.; Broers, J. L.; Leurs, R.; Bruggeman, C. A.; Smit, M. J.; Vink, C. The rat cytomegalovirus R33-encoded G protein-coupled receptor signals in a constitutive fashion. *J. Virol.* **2002**, *76*, 1328-1338.
72. Casarosa, P.; Gruijthuijsen, Y. K ; Michel, D.; Beisser, P. S.; Holl, J.; Fitzsimons, C. P.; Verzijl, D.; Bruggeman, C. A.; Mertens, T.; Leurs, R.; Vink, C.; Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded receptor UL33 differs from that of its rat cytomegalovirus homolog R33 by promiscuous activation of G proteins of the Gq, Gi, and Gs classes. *J Biol. Chem.* **2003**, *278*, 50010-50023.
73. Beisser, P. S.; Vink, C.; van Dam, J. G.; Grauls, G.; Vanherle, S. J. V.; Bruggeman, C. A. The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection. *J. Virol.*, **1998**, *72*, 2352–2363.
74. Streblow, D. N.; Kreklywich, C. N.; Smith, P.; Soule, J. L.; Meyer, C.; Yin, M.; Beisser, P.; Vink, C.; Nelson, J. A.; Orloff, S. L. Rat cytomegalovirus-accelerated transplant vascular sclerosis is reduced with mutation of the chemokine-receptor R33. Am. J. Transplant. 2005, 5, 436-442.
75. Kaptein, S. J. F.; Beisser, P. S.; Gruijthuijsen, Y. K.; Savelkouls, K. G. M.; van Cleef, K. W. R.; Beuken, E.; Grauls, G. E. L. M.; Bruggeman, C. A.; Vink, C. The rat cytomegalovirus R78 G protein-coupled receptor gene is required for production of infectious virus in the spleen. *J. Gen. Virol.* **2003**, *84*, 2517–2530.
76. Waldhoer, M.; Kledal, T. N.; Farrell, H.; Schwartz, T. W. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. *J. Virol.* **2002**, *76*, 8161-8168.
77. Margulies, B. J.; Gibson, W. The chemokine receptor homologue encoded by US27 of human cytomegalovirus is heavily glycosylated and is present in infected human foreskin fibroblasts and enveloped virus particles. *Virus Res.* **2007**, *123*, 57-71.
78. Kledal, T. N.; Rosenkilde, M. M.; Schwartz, T. W. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett.* **1998**, *441*, 209-214.
79. Billstrom, M. A.; Lehma, L. A.; Scott Worthen, G. Depletion of extracellulair RANTES during human cytomegalovirus infection of endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 163-167.
80. Casarosa, P; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R. Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
81. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first

nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
82. Chen, F.; Castranova, V.; Shi, X.; Demers, L. M. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **1999**, *45*, 7-17.
83. Bais, C.; Santomasso, B.; Coso, O.; Arvanitakis, L.; Geras-Raaka, E.; Gutkind, J. S.; Asch, A. S.; Cesarman, E.; Gershengorn, M. C. Mesri, E. A. G-protein-coupled receptor of Kaposi's sarcoma associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature* **1998**, *391*, 86-89.
84. Holst, P. J.; Rosenkilde, M. M.; Manfra, D.; Chen, S.-C.; Wiekowski, M. T.; Holst, B.; Cifire, F.; Lipp, M.; Schwartz, T. W. Tumorigenesis induced by the HHV8-encoded chemokine receptor requires ligand modulation of high constitutive activity. *J. Clin. Invest.* **2001**, *108*, 1789-1796.
85. Billstrom, M. A.; Johnson, G. L.; Avdi, N. J.; Worthen, G. S. Intracellular signaling by the chemokinereceptor US28 during human cytomegalovirus infection. *J. Virol.* **1998**, *72*, 5535-5544.
86. McLean, K. A.; Holst, P. J.; Martini, L.; Schwartz, T. W.; Rosenkilde, M. M. Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. *Virology* **2004**, *325*, 241-251.
87. Waldhoer, M.; Casarosa, P.; Rosenkilde, M. M.; Smit, M. J.; Leurs, R.; Whistler, J. L.; Schwartz, T. W. The carboxyl terminus of human cytomegalovirus-encoded 7 transmembrane receptor US28 camouflages agonism by mediating constitutive endocytosis. *J. Biol. Chem.* **2003**, 278, 19473-19482.
88. Pleskoff, O.; Treboute, C.; Alizon, M. The cytomegalovirus-encoded chemokine receptor US28 can enhance cell-cell fusion mediated by different viral proteins. *J. Virol.* **1998**, *72*, 6389-6397.
89. Pleskoff, O.; Treboute, C.; Belot, A.; Heveker, N.; Seman, M.; Alizon, M. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **1997**, *276*, 1874-1878.
90. Streblow, D. N.; Soderberg-Naucler, C.; Vieira, J.; Smith, P.; Wakabayashi, E.; Ruchti, F.; Mattison, K.; Altschuler, Y.; Nelson, J. A. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **1999**, *99*, 511-520.
91. Cinatl, J. Jr.; Vogel, J. U.; Kotchetkov, R.; Doerr, H. W. Oncomodulatory signals by regulatory proteins encoded by human cytomegalovirus: a novel role for viral infection in tumor progression. *FEMS Microbiol. Rev.* **2004**, *28*, 59-77.
92. Maussang, D.; Verzijl, D.; van Walsum, M.; Leurs, R.; Holl, J.; Pleskoff, O.; Michel, D.; van Dongen, G. A. M. S.; Smit, M. J. Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13068-13073.
93. Schall, T. J.; McMaster, B. E.; Dairaghi, D. J. Reagents and methods for the diagnosis of CMV dissemination. World (PTC) Patent WO0217969, **2002**.
94. Schall, T. J.; McMaster, B. E.; Dairaghi, D. J. Modulators of US28. World (PTC) Patent WO0217900, **2002**.
95. Laruelle, M.; Abi-Dargham, A, Dopamine as the wind of the psychotic fire: new evidence from brain imaging studies. *J Psychopharmacol.* **1999**, *13*, 358-371.
96. McMaster, B. E.; Schall, T. J.; Penfold, M.; Wright, J. J.; Dairaghi, D. J. Bicyclic compounds as inhibitors of chemokine binding to US28. World (PTC) Patent WO03018549, **2003**.
97. Liang, M.; Rosser, M.; Ng, H. P.; May, K.; Bauman, J. G.; Islam, I.; Ghannam, A.; Kretschmer, P. J.; Pu, H.; Dunning, L.; Snider, R. M.; Morrissey, M. M.; Hesselgesser, J.; Perez, D.; Horuk, R. Species selectivity of a small molecule antagonist for the CCR1 chemokine receptor. *Eur. J. Pharmacol*. **2000**, *389*, 41–49.
98. McMaster, B. E.; Schall, T. J.; Penfold, M.; Wright, J. J.; Dairaghi, D. J. Arylamines as inhibitors of chemokine binding to US28. World (PTC) Patent WO03020029, **2003**.
99. Hesselgesser, J.; Ng, H. P.; Liang, M.; Zheng, W.; May, K.; Bauman, J. G.; Monahan, S.; Islam, I.; Wei, G. P.; Ghannam, A.; Taub, D. D.; Rosser, M.; Snider, R. M.; Morrissey, M. M.; Perez, H. D.; Horuk, R. Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J. Biol. Chem.* **1998**, *273*, 15687-15692.
100. Mirzadegan, T.; Diehl, F.; Ebi, B.; Bhakta, S.; Polsky, I.; McCarley, D.; Mulkins, M.; Weatherhed, G. S.; Lapierre, J. M.; Dankwardt, J.; Morgans, D., Jr.; Wilhelm, R.; Jarnagin, K. Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokin receptor motif within the helical bundle. *J. Biol. Chem.* **2000**, *275*, 25562-25571.
101. Seibert, C.; Ying, W.; Gavrilov, S.; Tsamis, F.; Kuhmann, S. E.; Palani, A.; Tagat, J. R.; Clader, J. W.; McCombie, S. W.; Baroudy, B. M.; Smith, S. O.; Dragic, T.; Moore, J. P.; Sakmar, T. P. Interaction of small molecule inhibitors of HIV-1 entry with CCR5. *Virology* **2006**, *349*, 41-54.
102. Rosenkilde, M. M.; Schwartz, T. W. GluVII:06 – a higly conserved and selective anchor point for non-peptide ligands in chemokine receptors. *Curr. Top. Med. Chem.* **2006**, *6*, 1319-1333.

Chapter 2

Synthesis and structure-activity relationships of the first nonpeptidergic inverse agonists for the human cytomegalovirus-encoded chemokine receptor US28

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Abstract

US28 is a human cytomegalovirus (HCMV) encoded G protein-coupled receptor that signals in a constitutively active manner. Recently, we identified **1** {5-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile} as the first reported nonpeptidergic inverse agonist for a viral-encoded chemokine receptor. Interestingly, this compound is able to partially inhibit the viral entry of HIV-1. In this study we describe the synthesis of **1** and several of its analogues and unique structure-activity relationships for this first class of small-molecule ligands for the chemokine receptor US28. Moreover, the compounds have been pharmacologically characterized as inverse agonists on US28. By modification of lead structure **1**, it is shown that a 4-phenylpiperidine moiety is essential for affinity and activity. Other structural features of **1** are shown to be of less importance. These compounds define the first SAR of ligands on a viral GPCR (US28) and may therefore serve as important tools to investigate the significance of US28-mediated constitutive activity during viral infection.

Introduction

Human cytomegalovirus (HCMV) is a widespread β-herpesvirus that, like other herpes viruses, persists during the lifetime of the host.[1] Infection of individuals with HCMV is common, reaching a seroprevalence of 50-90% in adults, and is normally without clinical symptoms.[2] However, in immunologically immature or immunocompromised hosts, like premature neonates, acquired immunodeficiency syndrome (AIDS) patients, and transplant recipients, the virus can cause serious and even life-threatening disease.[1] Infection with HCMV is furthermore suggested to be associated with vascular diseases such as arterial restenosis, atherosclerosis and chronic allograft rejection.[3-5]

The genome of human cytomegalovirus encodes four G protein-coupled receptors (GPCRs), namely the open reading frames (ORFs) UL33, UL78, US27, and US28.[6] Two of these GPCRs, UL33 and UL78, have counterparts in the genome of both rat CMV (R33 and R78, respectively) and mouse CMV (M33 and M78, respectively), whereas US27 and US28 are specific for HCMV.[7] US28 shows high homology with β mammalian chemokine receptors, binds several CC chemokines with high affinity,[8-10] and is able to sequester CC chemokines from the extracellular environment via endocytosis.[11,12] This feature appears to be a putative strategy of the virus to escape immune surveillance by reducing the immune response to sites of HCMV infection.[13] US28 is able to bind not only CC chemokines but also the membrane-bound CX_3C chemokine CX3CL1/fractalkine, which has been suggested to play a role in the cell to cell transfer of HCMV.[14] Furthermore, upon binding with the CC chemokines CCL2/MCP-1 and CCL5/RANTES, US28 induces migration of vascular smooth muscle cells, which could provide the molecular basis of the role of HCMV in vascular diseases. The migration of smooth muscle cells can also be exploited by HCMV to enhance dissemination of the virus through the body.[15] Similar to other chemokine receptors, such as CCR5 and CXCR4, US28 can act as a co-receptor for human immunodeficiency virus-1 (HIV-1) entry when coexpressed with CD4.[16] Taken together, US28 is considered as an interesting drug target.

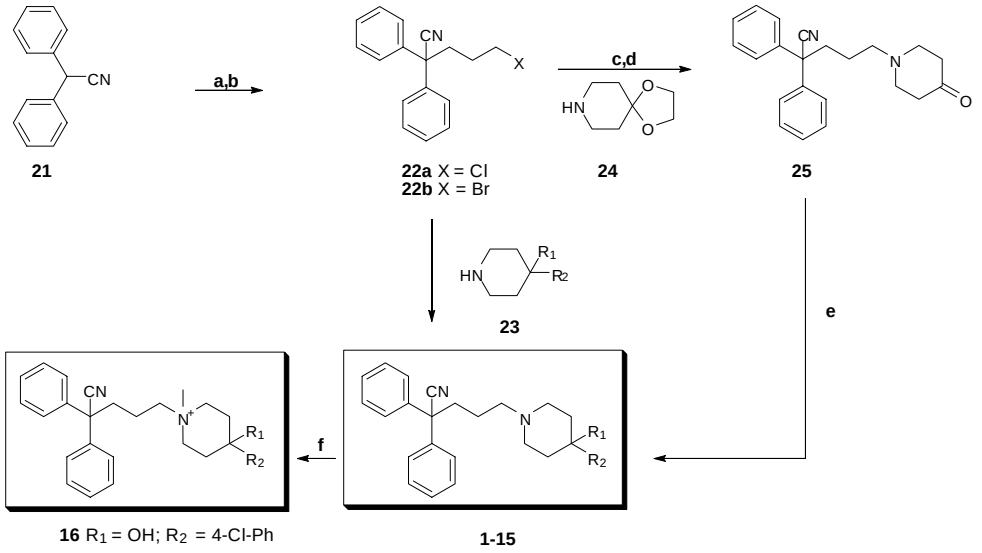
In previous studies we have shown that in transiently transfected COS-7 cells as well as in HCMV-infected cells US28 constitutively activates phospholipase C and the transcription factor NF-κB in an agonist-independent manner.[17,18] Interestingly, the CC chemokines CCL2 and CCL5 do not affect the basal US28 signaling and act as neutral antagonists, whereas the CX3C-chemokine CX3CL1 partially inhibits the constitutive signaling and acts as a partial inverse agonist. Constitutive activity is suggested to be a general characteristic of viral-encoded GPCRs. Besides the HCMV-encoded receptor US28, other viral-encoded receptors also signal in the absence of any ligand, namely the HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV) encoded GPCR ORF74[19] and the M33 gene family members encoded by MCMV, HCMV, and RCMV.[18,20,21] Although the biological relevance of constitutive activity has not been completely elucidated yet, it is believed to play an important role in the pathogenesis of virus infection. This is demonstrated for ORF74, which is a viral oncogene resulting in the development of Kaposi's sarcoma-like lesions in transgenic mice.[22,23]

Inverse agonists are able to inhibit the constitutive activation of GPCRs. The identification of such molecules for viral-encoded GPCRs can be an important tool to investigate the significance of constitutive activity and the influence of these receptors in viral infection. Recently, we identified **1** as a small nonpeptidergic molecule that acts as a full inverse agonist on US28. Furthermore, this compound is able to inhibit 60% of the US28-mediated HIV entry in cells at a concentration of 1 μM.[18] This molecule has been previously reported as an antagonist on the human chemokine receptor CCR1[24] and was screened on US28 because of the sequence homology of this viral receptor with CCR1 (33% identity).[10] In this study, the synthesis of **1** and various analogues is reported and we present the first structure-activity relationships for the interaction of these ligands with US28.

Chemistry

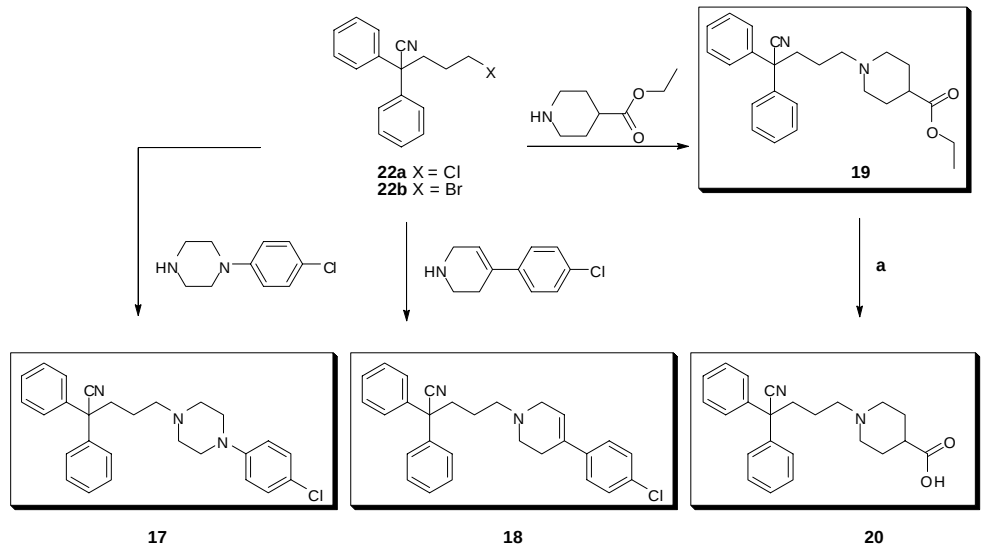
For the synthesis of compounds **1**-**20** the following synthetic routes were applied (Schemes 1 and 2). 2,2-Diphenylacetonitrile **21** was deprotonated with NaH at 0 °C followed by a reaction with the appropriate 1,ω-dihalopropanes to yield intermediates **22a** or **22b**.[24,25] Compounds **2**, **13**, **17**, and **18** were synthesized

by stirring intermediate **22a** in DMF at 90 °C in the presence of the corresponding amine and an excess of K_2CO_3 (method A).[24] The yields after purification were in the range of 15-50%. The reaction conditions were optimized by reacting intermediate **22a** or **22b** with the corresponding piperidine **23** in the presence of NaI, Na_2CO_3, and CH_3CN at reflux temperature (method B)[26] to give the desired products in yields ranging from 23% to 79%.



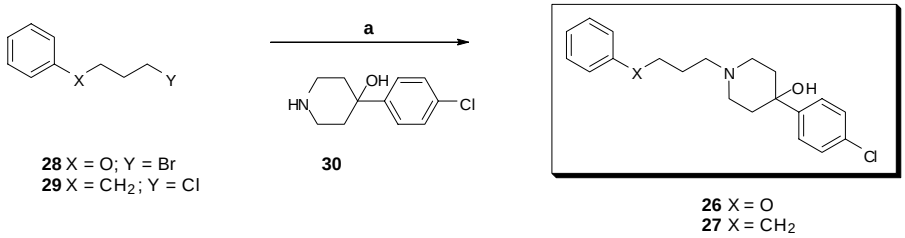
Scheme 1. Synthetic pathway for the synthesis of **1**-**16**. Reagents and conditions: (a) NaH, DMF, 0 °C; (b) 1,ω-Dihalopropane; (c) DMF, K_2CO_3, 90 °C or NaI, Na_2CO_3, CH_3CN, reflux; (d) 6 N HCl, MeOH; (e) R_2MgBr, THF, 0 °C or *n*-BuLi, THF, 0 °C for R_2 = *n*-butyl; (f) MeI, DCM.

For compounds **5**-**10** the appropriate piperidines were not commercially available. Consequently, **5-10** were synthesized according to literature procedures (Scheme 1, method C).[25] Intermediate **22** was reacted with protected piperidone **24**, followed by the deprotection with HCl to give ketone **25**. This was treated with the appropriate Grignard reagents to yield compounds **5-9**, whereas the reaction with *n*-BuLi afforded compound **10**.[25] The yields of products **5-10** were moderate to low, ranging from 5% to 42%, because of low yields in the recrystallization step.



Scheme 2. Synthetic pathway for the synthesis of **17**-**20**. Reagents and conditions: (a) DMF, K_2CO_3, 90 °C or NaI, Na_2CO_3, CH_3CN, reflux; (b) 2M NaOH, MeOH, reflux.

4-(Diphenylmethylene)piperidine, which was used for the synthesis of compound **15**, was obtained by an acid-catalyzed dehydratation of diphenyl(piperidin-4-yl)methanol with TFA.[27] The quaternary ammonium salt **16** was synthesized by the methylation of the piperidine nitrogen atom of **1** with methyl iodide.[25] Target compound **19** was synthesized following method B. The ethyl ester moiety of **19** was hydrolyzed in high yield with 2 M NaOH, resulting in the carboxylic acid derivative **20** (Scheme 2). Compounds **26** and **27** were prepared via the reaction of intermediates **28** and **29** with 4-(4-chlorophenyl)-4-piperidinol **30** (Scheme 3).



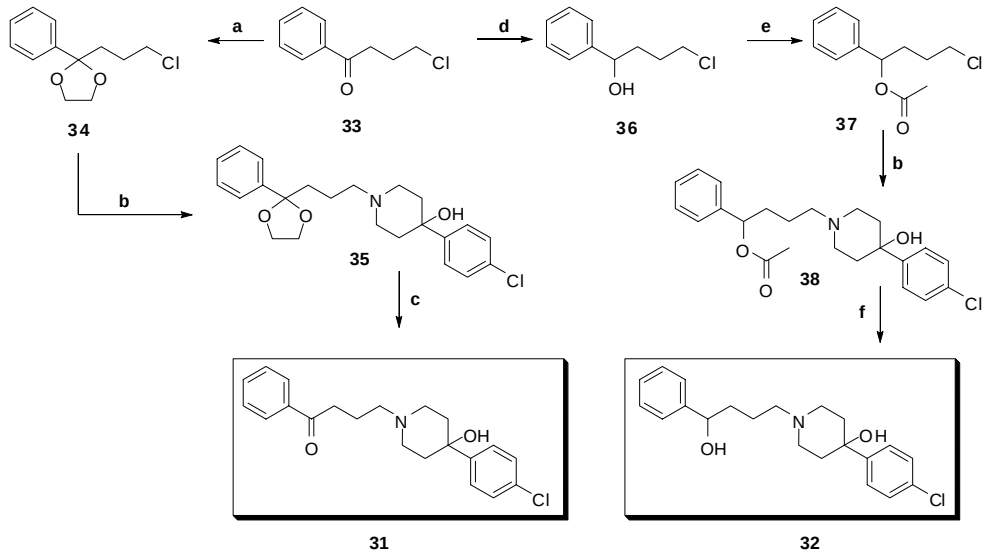
28 X = O; Y = Br
29 X = CH_2; Y = Cl

30

26 X = O
27 X = CH_2

Scheme 3. Synthetic pathway for the synthesis of **26**-**27**. Reagents and conditions: (a) NaI, Na_2CO_3, CH_3CN, reflux.

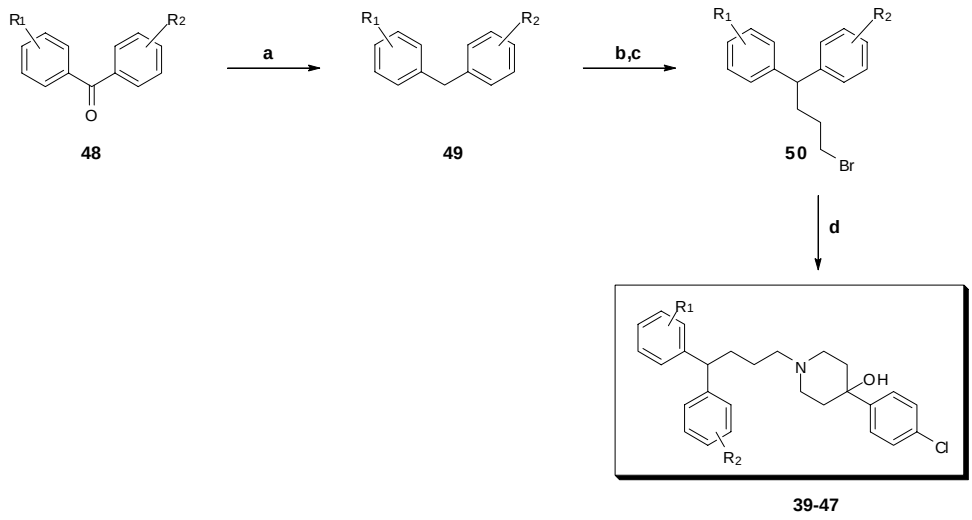
Compounds **31** and **32** were synthesized as depicted in Scheme 4. Butyrophenone derivative **31** was synthesized from commercially available reagents as earlier described.[28] The carbonyl group of **33** was ketalized with ethylene glycol in the presence of *p*-toluenesulphonic acid to afford the protected intermediate **34**. Alkylation of piperidine **30** with **34** and deprotection of the carbonyl group with HCl resulted in product **31**. For the synthesis of compound **32** 4-chloro-1-phenylbutan-1-one **33** was reduced with $NaBH_4$, resulting in alcohol **36**. Subsequently, the alcohol group was protected with acetyl chloride to give intermediate **37**, which was reacted with 4-(4-chlorophenyl)-4-piperidinol **30** resulting in compound **38**. Deprotection with NaOH afforded target compound **32**.



Scheme 4. Synthetic pathway for the synthesis of **31**-**32**. Reagents and conditions: (a) Ethylene glycol, *p*-toluene sulphonic acid monohydrate, toluene, reflux; (b) **30**, DMF, K_2CO_3, 90 °C or 30, NaI, Na_2CO_3, CH_3CN, reflux; (c) HCl, MeOH, reflux; (d) $NaBH_4$, EtOH, 0 °C; (e) acetylchloride, Et_3N, Et_2O; (f) 10% NaOH, MeOH, reflux.

Compounds **39**-**47** were synthesized according to the procedure shown in Scheme 5 (method D). Diphenylmethanes, which were not commercially available, were obtained from benzophenones **48** in the presence of $AlCl_3$ as a Lewis acid and a hydride donor ($NaBH_4$ or *tert*-butylamineborane).[29,30] Intermediates **49** were deprotonated with *n*-BuLi in THF, and the resulting

lithium salts reacted with 1,3-dibromopropane to afford bromides **50** in low yields.[31,32] The conversion of 4-methoxydiphenylmethane (**49**, R_1 = 4-OCH_3; R_2 = H) and 3,4-dichlorodiphenylmethane (**49**, R_1 = 3,4-Cl_2; R_2 = H) into the corresponding sodium salts or lithium salts with, respectively, $NaNH_2$ in liquid NH_3 or *n*-BuLi in THF was not successful. However, deprotonation of 3,4-dichlorodiphenylmethane was achieved with LDA in the presence of HMPA. For the deprotonation of 4-methoxydiphenylmethane the base strength of *n*-BuLi was not sufficient and a mixture of *n*-BuLi and potassium *tert*-butoxide in THF was used. Piperidine **30** was alkylated with intermediate **50** in CH_3CN in the presence of NaI and Na_2CO_3 to give the desired products **39**-**47**.



Scheme 5. Synthetic pathway for the synthesis of **39**-**47**. Reagents and conditions: (a) $AlCl_3$, $NaBH_4$, THF, 0 °C; for R_1 = 3,4-Cl_2, R_2 = H: $AlCl_3$ $(CH_3)_3CNH_2.BH_3$, THF, 0 °C; (b) *n*-BuLi, THF, -20 °C; for R_1 = 3,4-Cl_2, R_2 = H: LDA, HMPA, THF, -78 °C; for R_1 = 4-OCH_3, R_2 = H: *n*-BuLi, $(CH_3)_3COK$, -100 °C, THF; (c) 1-Bromo-3-chloropropane; (d) **30**, NaI, Na_2CO_3, CH_3CN, reflux.

Results and discussion

To identify a potential inverse agonist acting on US28, several GPCR-directed ligands were screened for their ability to modulate the basal signaling of this receptor. This resulted in the identification of compound **1** as the first nonpeptidergic inverse agonist acting on US28, as reported in an earlier paper describing US28 pharmacology.[18] It is noted that compound **1** has previously

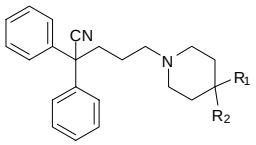
been reported as a potent antagonist for the human chemokine receptor CCR1, but it has no effects on the human chemokine receptors CCR5, CCR2, and CXCR4. Additionally, the selectivity of **1** was tested by screening against a number of human GPCRs and an at least 250-fold selectivity for the CCR1 chemokine receptor was observed. As expected, the only cross-reactivity of compound **1** was demonstrated for several biogenic amine neurotransmitter receptors.[24] Recently, it was also shown that this molecule binds to the human chemokine receptor CCR3 with micromolar affinity.[33] In all, we consider **1** to be an unique and interesting lead structure for the development of ligands directed toward the new class of viral GPCRs and a good tool to study viral GPCRs in a variety of assays with HCMV-infected cells.

Thus, compound **1** was used as a starting point for lead optimization, and several analogues were synthesized in order to investigate the first structure-activity relationship for inverse agonism on US28. All the synthesized compounds were evaluated for their potential to dose-dependently displace [^{125}I]CCL5 binding in COS-7 cells expressing US28. The effect on the US28-mediated constitutive inositol phosphate production in transiently expressed COS-7 cells was investigated for a selection of compounds. It is evident from the results in Tables 1-4 that the IC_{50} and EC_{50} values obtained from the binding assay and the functional assay, respectively, correlate well with each other. To determine the specificity of action the selected compounds were also tested on the constitutively active KSHV-encoded GPCR ORF74. The observed inositol phosphate production in ORF74 expressing cells was not affected up to a concentration of 10 μM (data not shown), indicating that the influence of these compounds on the inositol phosphate production was selective for US28.

To study the importance of the 4-chlorophenyl-4-hydroxypiperidine moiety, various substituted piperidine moieties were synthesized (Table 1). The influence of the substitution pattern of the phenyl ring was investigated by the substitution of the *p*-chlorosubstituent of **1** for substituents with different electronic and lipophilic properties in compounds **2**-**7**. Interestingly, all the compounds with variations in the para position, including the unsubstituted analogue **2**, were found to be less potent. The introduction of an additional

substituent at the meta position (*m*-trifluoro or *m*-chloro substituents in **3** and **6**, respectively) did not result in compounds with a higher affinity.

Table 1. Chemical structures and pharmacological properties of compounds **1**-**14** and **19**-**20** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.



no.	VUF	R_1	R_2	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274	OH	4-Cl-phenyl	9.3 (8.7 -10.0)	3.2 (2.5 – 4.0)
2	5660	OH	phenyl	35.5 (26.9 – 46.8)	28.2 (26.3 – 30.2)
3	5930	OH	3-CF_3-4-Cl-phenyl	45.7 (38.9 – 53.7)[c]	n.d.
4	5931	OH	4-Br-phenyl	19.5 (15.8 – 24.0)[c]	n.d.
5	5753	OH	4-OCH_3-phenyl	72.4 (55.0 – 95.5)	28.8 (25.7 – 32.4)[c]
6	5754	OH	3,4-Cl_2-phenyl	38.0 (28.2 – 51.3)	n.d.
7	5764	OH	4-CH_3-phenyl	60.3 (39.8 – 91.2)	n.d.
8	5786	OH	benzyl	63.1 (50.1 – 79.4)	n.d.
9	5787	OH	methyl	> 100	> 100
10	5765	OH	*n*-butyl	> 100	> 100
11	5744	OH	H	> 100	> 100
12	5929	CN	phenyl	> 100	> 100
13	5662	H	4-Cl-phenyl	10.5 (8.1 – 13.5)	13.8 (13.5 – 14.1)
14	5720	H	$C(O)NH_2$	>100[c]	> 100[c]
19	5719	H	COOEt	34.7 (26.3 – 45.7)	> 100
20	5718	H	COOH	> 100[c]	> 100

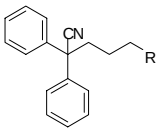
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Result of two independent experiments. n.d. = not determined.

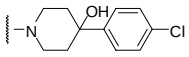
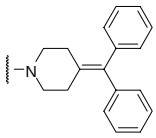
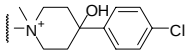
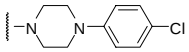
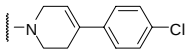
For the human chemokine receptor CCR1 there is a 2-fold improvement in *K*i value when *p*-chloro is replaced by a *p*-bromo substituent,[25] but on the viral-encoded receptor US28 the bromo substitution in **4** causes a 2-fold decrease in binding affinity. The binding affinities of the unsubstituted analogue **2** and compound **8**, with a benzyl group at the 4-position of the piperidine ring, were comparable, but both affinities were lower than that of lead compound **1**. Apparently, the introduction of an additional methylene group has no positive effect on the affinity of the compound. Removal of the 4-chlorophenyl group in compound **11** or substitution of this group by a CH_3 group in **9** or an *n*-butyl chain in **10** resulted in complete loss of affinity. This suggests that a phenyl ring at the 4-position is of importance.

Next, the role of the 4-hydroxy group was studied in compounds **12** and **13**. It is noted that the 4-chlorophenyl-4-hydroxypiperidine moiety is a structural motif that is also present in, for example, haloperidol. For this antipsychotic drug it is known that the 4-hydroxy group is responsible for the conversion to potentially neurotoxic metabolites.[34,35] In this respect, it is interesting to see that the affinity and activity on US28 did not change significantly after removal of the 4-hydroxy group in **13**. Substitution of the hydroxy group of **2** into a nitrile group in compound **12** resulted in a complete loss of both affinity and activity. Next to this, substitution of the 4-position of the piperidine ring with an amide (**14**) or carboxylic acid (**20**) function caused a complete loss of affinity and efficacy, while an ethyl ester at this position (**19**) resulted in a more than 3-fold loss of affinity compared to lead compound **1**.

The influence of the piperidine ring was investigated by the synthesis of compounds **15-18** (Table 2). Previously, we described that the basic nitrogen atom of the piperidine ring of **1** probably has an important interaction with a glutamic acid residue in transmembrane 7 (Glu^{277}).[18] For this reason, the nitrogen atom at this position was maintained. But substitution of the piperidine ring into a piperazine ring in **17** or tetrahydropyridine moiety in **18** abolished activity. The loss of activity of both compounds might be due to a change in conformation of the 4-chlorophenyl ring through which the interaction of the aromatic moiety with the receptor is lost.

Table 2. Chemical structures and pharmacological properties of compounds **1** and **15-18** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.



no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		9.3 (8.7 -10.0)	3.2 (2.5 – 4.0)
15	5875		> 100	> 100
16	4999		> 100	> 100
17	5658		> 100	> 100
18	5664		> 100	> 100

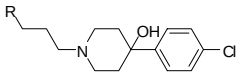
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

Introduction of the bulky 4-(diphenylmethylene)piperidine moiety of analogue **15** was detrimental for both affinity and potency. Methylation of the piperidine nitrogen atom of compound **1** gave quaternary ammonium salt **16**. Interestingly, this variation resulted in complete loss of binding affinity and activity on US28. For the human chemokine receptor CCR1 this modification resulted in a more than 6-fold increase in binding affinity,[25] clearly illustrating selectivity of compound **16** in favor of the CCR1 chemokine receptor. This indicates that there is a difference in structure-activity relationships of these compounds for both receptors, like that shown earlier in this section for compound **4**. Taken together, several analogues were synthesized to optimize the piperidine motif in the molecule, but all the variations in this part of the molecule resulted in a loss of affinity and potency.

Next, the influence of the diphenylacetonitrile moiety was investigated (Tables 3 and 4). The influence of the two phenyl rings was investigated by removal of one of the phenyl rings in compound **27** or replacement by more hydrophilic groups, namely a carbonyl group in compound **31** and a hydroxy group in

compound **32**. Previously, it was suggested that a bulky lipophilic moiety is important at this position,[18] but we observed that removal of a phenyl ring as in **27** resulted in a binding affinity comparable to that of lead compound **1**. In contrast, the more hydrophilic groups in **31-32** resulted in loss of effect. Also the introduction of an isosteric oxygen atom in the butyl chain in compound **26** did not improve affinity.

Table 3. Chemical structures and pharmacological properties of compounds **1, 26-27, 31-32** and **39** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.

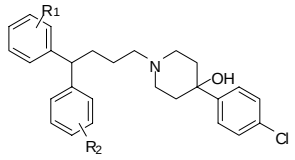


no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		9.3 (8.7 -10.0)	3.2 (2.5 – 4.0)
39	5667		6.5 (6.0 – 6.9)	4.2 (3.1 – 5.6)
26	5714		61.7 (57.5 – 66.1)[c]	n.d
27	5746		14.8 (13.8 – 15.8)	n.d.
31	5666		> 100[c]	n.d.
32	(±)-5715		> 100	> 100

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Result of two experiments. n.d. = not determined.

Interestingly, the nitrile group of compound **1** is not essential in the molecule because its removal, resulting in compound **39**, did not affect the activity of the compound (Table 4). Compounds **40**-**42** and **44-47** possess a chiral center as a result of the substitution in one or both of the phenyl rings. The two enantiomers were not separated, and the activity was thus determined for the racemic mixtures. Compounds **40-42** and **46** were synthesized to determine the effects of the introduction of para substituents with different electronic and lipohilic properties in the two aromatic rings. The variations in the substitution pattern in the para position did not significantly influence the activity of the compounds. Also, the introduction of an additional *p*-chloro atom (compound **43**) did not increase the affinity of the compound for US28.

Table 4. Chemical structures and pharmacological properties of compounds **39-47** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.



no.	VUF	R_1	R_2	IC_{50} (μM)[a]	EC_{50} (μM)[b]
39	5667	H	H	6.5 (6.0 – 6.9)	4.2 (3.1 – 5.6)
40	(±)-5857	4-OCH_3	H	15.5 (13.2 – 18.2)	n.d
41	(±)-5858	3,4-Cl_2	H	15.8 (14.5 – 17.4)	n.d.
42	(±)-5859	4-Cl	H	17.0 (16.6 – 17.4)	n.d.
43	5860	4-Cl	4-Cl	17.4 (16.2 – 18.6)	n.d.
44	(±)-5861	2-CH_3	H	5.5 (5.0 – 6.0)	9.8 (9.5 – 10.0)[c]
45	(±)-5862	3-CH_3	H	11.5 (8.7 – 15.1)	n.d.
46	(±)-5863	4-CH_3	H	30.9 (27.5 – 34.7)	11.5 (11.0 – 12.0)[c]
47	(±)-5864	4-Cl	4-phenyl	> 100	> 100

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Result of two independent experiments. n.d. = not determined.

The differences in binding affinities between the methyl-substituted analogues **44-46** and the unsubstituted analogue **39** reveal a slight preference for *o*-methyl substitution. This could be caused by the restricted conformational freedom of the substructure in compound **44**. Methyl substitution at the ortho position of a similar aromatic benzhydryl sytem was previously described for a series of histamine H_1 receptor antagonists. It is suggested that the *o*-methyl analogue of diphenhydramine has a markedly reduced antihistamine activity because the methyl group in that position prevents the molecule from adopting the conformation necessary for antihistamine activity.[36] The influence of a bulky aromatic substituent in the para position was investigated by the synthesis of analogue **47**. The additional *p*-phenyl group resulted in a loss of affinity and potency, and it seems that bulky substituents are not allowed at this position.

Conclusions

This is the very first study of small molecules acting as inverse agonists on US28. We described the synthesis of compound **1** and different analogues, which were evaluated for their binding affinity by displacement of [^{125}I]CCL5. Moreover, a selection of compounds was evaluated for their functionality by measuring the effect on the US28-mediated constitutive inositol phosphate production. This resulted in unique structure-activity relationships for the first small molecule US28 receptor ligands.

With this study we have acquired important new insights about the first inverse agonists acting on US28. These structural insights will be used to develop new compounds with an improved affinity and potency as well as a better selectivity for this receptor. These small molecules may serve as important tools to investigate the significance of the constitutive activity and the role of US28 during viral infection.

Experimental section

General procedures. ^{1}H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer with tetramethylsilane as internal standard. J.T. Baker silica gel was used for flash chromatography. Mass spectra were recorded on a Finnigan MAT-90 mass spectrometer. Melting points were measured on an Electrothermal IA9200 apparatus and were uncorrected. Elemental analyses were performed by Microanalytisches Labor Pascher, Remagen-Bandorf, Germany and the results were within ± 0.4% of the theoretical values unless otherwise stated. The solvents were dried according to standard procedures. All reactions were performed under an atmosphere of dry nitrogen.

General method A. 5-(4-(4-Chlorophenyl)-piperazin-1-yl)-2,2-diphenypentanenitrile (17). A solution of **22a** (0.51 g, 1.88 mmol), which was synthesized according to literature procedure,[24] 4-chlorophenylpiperazine (0.45 g, 1.67 mmol), and K_2CO_3 (3.53 g, 25.6 mmol) in DMF (25 mL) was stirred overnight at 90 °C. The solvent was removed in vacuo, and the residue was dissolved in water (25 mL) followed by an extraction with EtOAc (3 x 10 mL). The combined organic layers were washed with water (2 x 10 mL), dried over anhydrous $MgSO_4$, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0-100% EtOAc in DCM) to give 107 mg (15%) of **17** as a light-yellow solid. Mp: 126.2-126.7 °C. ^{1}H NMR ($CDCl_3$): δ 1.52-1.71 (m, 2H), 2.37-2.51 (m, 8H), 3.11 (m, 4H), 6.80 (d, *J* = 9.0 Hz, 2H), 7.15-7.40 (m, 12H). MS ESI *m/z*: 431.0 $(M+H)^+$. Anal. ($C_{27}H_{28}ClN_3$) C, H, N.

General method B. 5-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenyl-pentanenitrile (1). A solution of **22b** (0.94 g, 3.00 mmol), which was synthesized according to literature procedure,[25] 4-(4-chlorophenyl)-4-piperidinol **30** (0.67 g, 3.15 mmol), NaI (0.50 g, 3.31 mmol), and Na_2CO_3 (0.64 g, 6.04 mmol) in CH_3CN (25 mL) was refluxed overnight. The solvent was removed in vacuo and the residue was diluted with water (50 mL) followed by an extraction with DCM (3 x 30 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (0-100% EtOAc in DCM) and crystallization from EtOAc gave 1.04 g (78%) of **1** as a white solid. Mp: 133.6-135.2 °C. ^{1}H NMR ($CDCl_3$): δ 1.55-1.70 (m, 4H), 1.93-2.12 (m, 3H), 2.28-2.50 (m, 6H), 2.61-2.78 (m, 2H), 7.18-7.48 (m, 14H). MS ESI *m*/*z*: 446.9 $(M+H)^+$. Anal. ($C_{28}H_{29}N_2ClO$·0.21EtOAc) C, H, N.

General method C. 5-(4-(4-Methoxyphenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenyl-pentanenitrile (5). A solution of (4-methoxyphenyl)magnesium bromide[37] (7.7 mL, 0.5 M in dry Et_2O, 3.6 mmol) was cooled to 0 °C, and a solution of **25** (1.00 g, 3.00 mmol), which was synthesized according to literature procedure,[25] dissolved in THF (15 mL) was added in one portion via a dropping funnel. The reaction mixture was stirred at room temperature for 4 h, and the reaction was quenched with water (30 mL). This solution was acidified with 1 N HCl, stirred for 15 min and basified with K_2CO_3. The product was extracted with EtOAc (3 x 100mL) and the combined organic layers were washed with water (2 x 100 mL) and brine (100 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by flash chromatography (EtOAc) afforded 780 mg (59%) of **5**. Recrystallization of one batch from Et_2O gave 190 mg (14%) of **5** as white crystals. Mp: 84.7-86.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.69-1.74 (m, 5H), 2.02-2.18 (m, 2H), 2.35-2.50 (m, 6H), 2.68-2.79 (m, 2H), 3.78 (s, 3H), 6.86 (d, *J* = 8.9 Hz, 2H), 7.24-7.42 (m, 12H). MS ESI *m*/*z*: 441.4 $(M+H)^+$. Anal. (C29H32N2O2) C, H, N.

General method D. 4-(4-Chlorophenyl)-1-(4-phenyl-4-*o*-tolylbutyl)piperidin-4-ol (44).
(i) A solution of 2-methylbenzophenone (5.34 g, 27.2 mmol) in THF (125 mL) was cooled to 0 °C, and subsequently $AlCl_3$ (10.06 g, 75.4 mmol) and $NaBH_4$ (5.26 g, 139 mmol) were added. The reaction mixture was heated and refluxed for 3 h. Next, the mixture was cooled to 0 °C and diluted carefully by the dropwise addition of water (100 mL). After separation of the organic layer, the water layer was extracted with EtOAc (3 x 100 mL). The combined organic layers were washed with water (3 x 150 mL) and brine (150 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography (hexane) gave 3.81 g (77%) of 2-methyldiphenylmethane as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 2.37 (s, 3H), 4.11 (s, 2H), 7.20-7.46 (m, 9H).
(ii) A solution of 2-methyldiphenylmethane (1.87 g, 10.3 mmol) in THF (20 mL) was cooled to -20 °C, and *n*-BuLi (6.40 mL, 1.6 M in hexane, 10.2 mmol) was added. The solution was stirred for 1 h and

added slowly to a solution of 1,3-dibromopropane (2.30 mL, 22.7 mmol) in THF (35 mL) at -76 °C. The mixture was allowed to warm to room temperature and stirred for 30 min. The solvent was evaporated under reduced pressure. Water (25 mL) was added to the residue, and the water layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water (3 × 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by flash chromatography (hexane) to give 0.81 g (26%) of bromide **50** (R_1 = 2-Me; R_2 = H) as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 1.87-2.00 (m, 2H), 2.18-2.30 (m, 2H), 2.34 (s, 3H), 3.44 (t, *J* = 6.6 Hz, 2H), 4.18 (t, *J* = 8.1 Hz, 1H), 7.18-7.42 (m, 9H).

(iii) **50** was reacted with 4-(4-chlorophenyl)-4-piperidinol **30** (0.70 g, 3.3 mmol) according to method B to give 834 mg (72%) of **44** as white crystals after recrystallization from EtOAc. Mp: 143.1-144.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.55-1.69 (m, 5H), 2.00-2.12 (m, 4H), 2.25 (s, 3H), 2.33-2.44 (m, 4H), 2.71-2.78 (m, 2H), 4.09 (t, *J* = 7.6 Hz, 1H), 7.07-7.33 (m, 11H), 7.41 (d, *J* = 8.8 Hz, 2H). MS ESI *m/z*: 434.4 $(M+H)^+$. Anal. ($C_{28}H_{32}ClNO$) C, H, N, Cl.

5-(4-Hydroxy-4-phenylpiperidin-1-yl)-2,2-diphenylpentanenitrile (2). Following method A using **22a** (0.51 g, 1.90 mmol) and 4-hydroxy-4-phenylpiperidine (0.34 g, 1.93 mmol) gave 319 mg (42%) of **2** as a white solid.[24] Mp: 73.6-74.7 °C. ^{1}H NMR ($CDCl_3$): δ 1.51-1.80 (m, 4H), 2.06-2.19 (m, 3H), 2.30-2.50 (m, 6H), 2.64-2.78 (m, 2H), 7.12-7.51 (m, 15H). MS ESI *m/z*: 411.3 $(M+H)^+$. Anal. ($C_{28}H_{30}N_2O \cdot 0.36H_2O$) C, H, N.

5-(4-(3-Chloro-4-trifluoromethylphenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (3). Using **22a** (0.65 g, 2.40 mmol) and 4-(4-chloro-3-(trifluoromethyl)phenyl)-4-piperidinol (0.56 g, 2.01 mmol) gave 0.93 g (90%) of the free base as a thick oil. This was dissolved in MeOH (5 mL), and subsequently 6 N HCl (0.15 mL) and Et_2O (20 mL) were added dropwise while stirring. The hydrochloride salt was isolated by filtration and recrystallized from Et_2O/MeOH to give 666 mg (67%) of **3** as a white solid. Mp: 231.2-234.0 °C (dec). ^{1}H NMR ($CDCl_3$): δ 1.61-1.82 (m, 5H), 2.07-2.26 (m, 2H), 2.31-2.53 (m, 6H), 2.70-2.89 (m, 2H), 7.24-7.46 (m, 13H). MS ESI *m/z*: 513.4 $(M+H)^+$. Anal. ($C_{29}H_{29}Cl_2N_2F_3O$) C, H, N.

5-(4-(4-Bromophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile (4). Following method B using **22a** (0.65 g, 2.39 mmol) and 4-(4-bromophenyl)-4-piperidinol (0.51 g, 2.01 mmol) gave 819 mg (83%) of **4** as a white solid. Mp: 131.6-132.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.61-1.82 (m, 5H), 2.07-2.26 (m, 2H), 2.31-2.53 (m, 6H), 2.70-2.89 (m, 2H), 7.24-7.46 (m, 14H). MS ESI *m/z*: 490.8 $(M+H)^+$. Anal. ($C_{28}H_{29}N_2BrO \cdot 0.34DCM$) C, H, N.

5-(4-(3,4-Dichlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile (6). Following method C using **25** (1.00 g, 3.00 mmol) and (3,4-dichlorophenyl)magnesium bromide[37] (3.3 mL, 1.1 M in dry Et_2O, 3.6 mmol) followed by purification by flash chromatography (0-100% EtOAc in

DCM) afforded 944 mg of **6**. Recrystallization from Et_2O gave 212 mg (15%) of **6** as white crystals. Mp: 102.0-103.4 °C. ^1H NMR ($CDCl_3$): δ 1.59-1.68 (m, 5H), 2.01-2.12 (m, 2H), 2.28-2.49 (m, 6H), 2.68-2.79 (m, 2H), 7.24-7.41 (m, 12H), 7.59 (d, *J* = 2.1 Hz, 1H). MS ESI *m*/*z*: 479.5 $(M+H)^+$. Anal. ($C_{28}H_{28}Cl_2N_2O$) C, H, N, Cl.

5-(4-Hydroxy-4-*p*-tolylpiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (7). Following method C using **25** (1.00 g, 3.00 mmol) and *p*-tolylmagnesium bromide[37] (3.6 mL, 1.0 M in dry Et_2O, 3.6 mmol) gave 280 mg of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. Recrystallization of one batch from Et_2O/MeOH gave 141 mg (10%) of **7** as a white solid. Mp: 225.0-226.3 °C. ^1H NMR ($CDCl_3$): δ 1.81-2.03 (m, 5H), 2.30 (s, 3H), 2.62-2.71 (m, 2H), 2.75-2.92 (m, 2H), 3.01-3.22 (m, 6H), 7.13 (d, *J* = 8.1 Hz, 2H), 7.24-7.45 (m, 12H), 12.15 (br s, 1H). MS ESI *m*/*z*: 425.4 $(M+H)^+$. Anal. ($C_{29}H_{33}ClN_2O$) C, H, N, Cl.

5-(4-Benzyl-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (8). Following method C using **25** (1.00 g, 3.00 mmol) and benzylmagnesium bromide[37] (3.4 mL, 1.1 M in dry Et_2O, 3.7 mmol) gave 147 mg of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. One batch was recrystallized from Et_2O/MeOH to give 86 mg (5%) of **8** as a white solid. Mp: 121.8-123.8 °C. ^1H NMR ($CDCl_3$): δ 1.51-1.65 (m, 3H), 1.90-1.99 (m, 2H), 2.38-2.51 (m, 2H), 2.59-2.65 (m, 2H), 2.80 (s, 2H), 2.89-3.00 (m, 4H), 3.14-3.22 (m, 2H), 7.16-7.38 (m, 15H), 12.02 (br s, 1H). MS ESI *m*/*z*: 425.5 $(M+H)^+$. Anal. ($C_{29}H_{33}ClN_2O \cdot 1.0H_2O$) C, H, N, Cl.

5-(4-Hydroxy-4-methylpiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (9). Following method C using **25** (1.00 g, 4.00 mmol) and methylmagnesium iodide[37] (2.8 mL, 1.3 M in dry Et_2O, 3.6 mmol) followed by purification by flash chromatography (0-7% EtOH in DCM) gave 500 mg of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. Recrystallization from Et_2O/MeOH gave 441 mg (42%) of **9** as a white solid. Mp: 192.7-193.5 °C. ^1H NMR ($CDCl_3$): δ 1.32 (s, 3H), 1.63-1.69 (m, 3H), 1.88-2.05 (m, 2H), 2.28-2.44 (m, 2H), 2.62 (t, *J* = 7.9 Hz, 2H), 2.90-3.21 (m, 6H), 7.24-7.43 (m, 10H), 11.88 (br s, 1H). MS ESI *m*/*z*: 349.4 $(M+H)^+$. Anal. ($C_{23}H_{29}ClN_2O$) C, H, N, Cl.

5-(4-Butyl-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (10). This compound was synthesized as described in the literature[25] and converted to the hydrochloride salt as described for **3**. One batch was recrystallized from Et_2O/MeOH to afford 304 mg (18%) of **10** as a white solid. Mp: 174.2-176.0 °C (dec). ^1H NMR ($CDCl_3$): δ 0.80-0.93 (t, *J* = 4.6 Hz, 3H), 1.18-1.40 (m, 4H), 1.48-1.71 (m, 5H) 1.88-2.08 (m, 2H), 2.28-2.43 (m, 2H), 2.60-70 (m, 2H), 2.89-3.22 (m, 6H), 7.24-7.44 (m, 10H), 12.02 (br s, 1H). MS ESI *m*/*z*: 391.4 $(M+H)^+$. Anal. ($C_{26}H_{35}ClN_2O$) C, H, N, Cl.

5-(4-Hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile (11). Following method B using **22b** (0.95 g, 3.01 mmol) and piperidin-4-ol (0.38 g, 3.75 mmol) followed by purification by flash

chromatography (10-50% MeOH in EtOAc) afforded 374 mg (37%) of **11** as a white solid. [25] Mp: 114.8-115.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.39-1.68 (m 5H), 1.80-1.91 (m, 2H), 1.95-2.11 (m, 2H), 2.31-2.44 (m, 4H), 2.59-2.71 (m, 2H), 3.59-3.72 (m, 1H), 7.22-7.42 (m, 10H). MS ESI *m/z*: 335.3 $(M+H)^+$. Anal. ($C_{22}H_{26}N_2O$) C, H, N.

1-(4-Cyano-4,4-diphenylbutyl)-4-phenylpiperidine-4-carbonitrile (12). Following method B using **22a** (0.65 g, 2.40 mmol) and 4-phenyl-piperidine-4-carbonitrile (0.45 g, 2.00 mmol) followed by crystallization from DCM/Et_2O gave 599 mg (66%) of **12** as a white solid. Mp: 137.5-138.7 °C. ^{1}H NMR ($CDCl_3$): δ 1.59-1.71 (m, 2H), 2.02-2.08 (m, 4H), 2.33-2.49 (m, 6H), 2.83-2.97 (m, 2H), 7.24-7.50 (m, 15H). MS ESI *m/z*: 420.7 $(M+H)^+$. Anal. ($C_{29}H_{29}N_3$) C, H, N.

5-(4-(4-Chlorophenyl)-piperidin-1-yl)-2,2-diphenylpentanenitrile (13). A solution of 4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine hydrochloride (1.00 g, 4.35 mmol) and 5% Pd/C (100 mg) in EtOH (25 mL) was stirred for 4 h under hydrogen. The solution was filtered and evaporated under reduced pressure. The residue was used without further purification, dissolved in DMF and reacted following method A using **22a** (0.63 g, 2.34 mmol). This afforded 954 mg (51%) of **13** as a white solid. Mp: 112.6-113.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.55-1.83 (m, 6H), 1.89-2.08 (m, 2H), 2.35-2.56 (m, 5H), 2.89-3.01 (m, 2H), 7.09-7.43 (m, 14H). MS ESI *m/z*: 430.0 $(M+H)^+$. Anal. ($C_{28}H_{29}ClN_2$) C, H, N.

1-(4-Cyano-4,4-diphenylbutyl)piperidine-4-carboxylic acid amide (14). Following method B using **22b** (0.94 g, 3.00 mmol) and piperidine-4-carboxylic acid amide (0.39 g, 3.00 mmol) gave 796 mg (73%) of **14** as an orange oil. ^{1}H NMR ($CDCl_3$): δ 1.61-2.52 (m, 13 H), 2.76-2.99 (m, 2H), 5.57 (br s, 2H), 7.24-7.45 (m, 10H). MS ESI *m/z*: 362.3 $(M+H)^+$. Anal. ($C_{25}H_{27}N_2O_2 \cdot 0.44DCM$) C, H, N.

5-(4-(Diphenylmethylene)piperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (15). Following method B using **22b** (0.92 g, 2.91 mmol) and 4-(diphenylmethylene)piperidine[27] (0.72 g, 2.86 mmol) followed by purification by flash chromatography (0-25% EtOAc in DCM) gave the free base as a thick oil. This was dissolved in a solution of MeOH (5 mL) and subsequently 6 N HCl (0.15 mL) and Et_2O (20 mL) were added dropwise while stirring. The hydrochloride salt was isolated by filtration and recrystallized from Et_2O/MeOH giving 863 mg (58%) of **15** as a white solid. Mp: 106.8-108.5. ^{1}H NMR (CH_3OH-d_4): δ 1.80-1.99 (m, 2H), 2.54-2.75 (m, 6H), 3.20-3.36 (m, 6H), 7.10-7.47 (m, 20H). MS ESI *m/z*: 483.9 $(M+H)^+$. Anal. ($C_{35}H_{36}Cl_2N_2 \cdot 1.0H_2O$) C, H, N.

4-(4-Chlorophenyl)-1-(4-cyano-4,4-diphenylbutyl)-4-hydroxy-1-methylpiperidinium iodide (16). Following the same procedure as described in the literature[25] afforded 206 mg (70%) of **16** as a white solid. Mp: 215.8 – 216.9 ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.70-2.00 (m, 4H), 2.06-2.31 (m, 2H), 2.35-2.60 (m, 2H), 3.18 (s, 3H), 3.30-3.50 (m, 2H), 3.52-3.90 (m, 4H), 4.94 (br s, OH), 7.12-7.43 (m, 14H). MS ESI m/z: 460.0 (M+H)+. Anal. ($C_{27}H_{32}ClN_2O$) C, H, N.

5-(4-(4-Chlorophenyl)-3,6-dihydro-2H-pyridin-1-yl)-2,2-diphenylpentanenitrile (18). Following method A using **22a** (0.81 g, 3.00 mmol) and 4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine **27** (0.69 g, 3.00 mmol) afforded 400 mg (31%) of **18** as a yellow solid. Mp: 142.1-142.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.56-1.78 (m, 2H), 2.41-2.62 (m, 8H), 3.06 (d, *J* = 3.1 Hz, 2H), 6.01 (m, 1H), 7.22-7.42 (m, 14H). MS ESI m/z: 427.9 (M+H)+. Anal. ($C_{28}H_{27}ClN_2$) C, H, N.

1-(4-Cyano-4,4-diphenylbutyl)-piperidine-4-carboxylic acid ethyl ester (19). Following method B using **22b** (1.26 g, 4.01 mmol) and piperidine-4-carboxylic acid ethyl ester **24** (0.79 g, 5.03 mmol) gave 1.65 g (84%) of **19** as an orange oil. ^{1}H NMR ($CDCl_3$): δ 1.25 (t, *J* = 8.0 Hz, 3H), 1.45–2.00 (m, 8H), 2.15-2.50 (m, 5H), 2.65-2.80 (m, 2H), 4.10 (m, 2H), 7.20-7.45 (m, 10H). MS ESI *m*/*z*: 391.4 $(M+H)^+$. Anal. ($C_{25}H_{30}N_2O_2$) C, H, N.

1-(4-Cyano-4,4-diphenylbutyl)piperidine-4-carboxylic acid hydrochloride (20). A solution of **19** (1.41 g, 3.61 mmol) and 2M NaOH (5 mL) in MeOH (30 mL) was refluxed for 3 h. The reaction mixture was allowed to cool to room temperature, evaporated in vacuo, diluted with water (30 mL), and extracted with Et_2O (1 x 30 mL). The water layer was acidified with 2 N HCl, and 1.23 g (86%) of **20** was isolated by filtration as a white solid. Mp: 231.0-233.1 °C. ^{1}H NMR (DMSO-d_6/D_2O): δ 1.40–1.80 (m, 4H), 1.75-2.15 (m, 2H), 2.40-2.65 (m, 3H), 2.70-2.94 (m, 2H), 2.96-3.18 (m, 2H), 3.27-3.39 (m, 2H), 7.29-7.54 (m, 10H). MS ESI *m*/*z*: 363.8 $(M+H)^+$. Anal. ($C_{23}H_{27}ClN_2O_2 \cdot 0.41H_2O$) C, H, N.

4-(4-Chlorophenyl)-1-(3-phenoxypropyl)piperidin-4-ol (26). Following method B using **28** (0.65 g, 3.00 mmol) and 4-(4-chlorophenyl)-4-piperidinol **30** (0.42 g, 2.00 mmol) gave a mixture of **26** and the quaternary product. The quaternary product was separated from **26** by fractional crystallization in $CHCl_3$. The filtrate was concentrated under reduced pressure and recrystallized from EtOAc to give 203 mg (29%) of **26** as white needles. Mp: 125.8-127.3 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.61-1.82 (m, 3H), 1.88-2.19 (m, 4H), 2.32-2.65 (m, 4H), 2.75-2.90 (m, 2H), 4.03 (t, *J* = 6.0 Hz, 2H), 6.80-6.95 (m, 3H), 7.15-7.34 (m, 4H), 7.36-7.45 (m, 2H). MS ESI *m*/*z*: 347.6 $(M+H)^+$. Anal. ($C_{20}H_{24}ClNO_2$) C, H, N.

4-(4-Chlorophenyl)-1-(4-phenylbutyl)piperidin-4-ol (27). Following method B using 1-chloro-4-phenylbutane **29** (0.49 mL, 2.98 mmol) and 4-(4-chlorophenyl)-4-piperidinol **30** (0.70 g, 3.30 mmol) gave 310 mg (35%) of **27** as a white solid.[33] Mp: 112.5-114.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.50-1.72 (m, 7H), 2.00-2.17 (m, 2H), 2.25-2.43 (m, 4H), 2.62 (t, *J* = 7.1 Hz, 2H), 2.72-2.85 (m, 2H), 7.15-7.31 (m, 7H), 7.42 (d, *J* = 8.6 Hz, 2H). MS ESI *m*/*z*: 344.3 $(M+H)^+$. Anal. ($C_{21}H_{26}ClNO$) C, H, N, Cl.

4-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-1-phenylbutan-1-one (31). (i) A solution of **33** (6 mL, 37.4 mmol), ethylene glycol (6 mL, 108 mmol) and *p*-toluenesulphonic acid monohydrate (0.50 g, 2.63 mmol) in toluene (400 mL) was refluxed overnight with azeotropic removal of water. The organic layer was washed with 5% $NaHCO_3$ (250 mL) and water (250 mL), dried over anhydrous Na_2SO_4, and evaporated in vacuo to give 9.69 g (98%) of **34** as an orange solid.

(ii) Following method B using **34** (0.72 g, 3.19 mmol) and 4-(4-chlorophenyl)-4-piperidinol **30** (0.57 g, 2.69 mmol) gave 1.28 g of a brown oil. This was dissolved in MeOH (15 mL), and concentrated HCl (1.4 mL) was added. The resulting reaction mixture was refluxed for 2 h, allowed to cool to room temperature, and evaporated in vacuo. The resulting brown oil was dissolved in EtOAc (25 mL) and washed with NH_4OH (5% solution in water, 2 x 15 mL) and water (2 x 5 mL). The organic layer was dried over anhydrous $MgSO_4$, rinsed with hexane, and evaporated in vacuo to give 522 mg (53%) of **31** as a white solid. Mp: 130.4-131.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.67-1.79 (m, 2H), 1.92-2.13 (m, 2H), 2.16-2.32 (m, 2H), 2.98-3.54 (m, 8H), 4.18 (br s, OH), 7.03-7.58 (m, 7H), 7.77 (d, J = 7.0 Hz, 2H). MS ESI m/z: 358.2 $(M+H)^+$. Anal. ($C_{21}H_{24}ClNO_2$·0.24hexane) C, H, N.

4-(4-Chlorophenyl)-1-(4-hydroxy-4-phenylbutyl)piperidin-4-ol (32). (i) **33** (3.94 g, 21.64 mmol) was dissolved in EtOH (25 mL), and $NaBH_4$ (0.42 g, 11.10 mmol) was added in small portions at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 1 h. After the addition of water (25 mL), the reaction mixture was extracted with Et_2O (3 x 25 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo to give 3.69 g (93%) of **36** as colourless oil, which was used without further purification.

(ii) Et_3N (8.5 mL, 61.2 mmol) and acetyl chloride (2.6 mL, 30.6 mmol) were added to a solution of **36** in Et_2O (50 mL). The reaction mixture was stirred at room temperature for 1 h, diluted with water (25 mL) and extracted with Et_2O (3 x 25 mL). The combined organic layers were washed with aqueous K_2CO_3 (25 mL), dried over anhydrous $MgSO_4$, and evaporated in vacuo to give 2.51 g (51%) of **37** as brown oil, which was used without further purification.

(iii) Following method B using **37** (0.68 g, 3.00 mmol) and 4-(4-chlorophenyl)-4-piperidinol **30** (0.70 g, 3.32 mmol) gave 1.24 g of **38**. This was dissolved in MeOH (25 mL), and 10% NaOH (5 mL) was added. The resulting reaction mixture was refluxed for 30 min, evaporated in vacuo and extracted with DCM (3 × 25 mL). The combined organic layers were dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by flash chromatography (0-10% MeOH in EtOAc) to give 449 mg (41%) of **32** as a white solid. Mp: 144.4-145.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.51-2.07 (m, 8H), 2.09-2.31 (m, 2H), 2.40-2.69 (m, 4H), 2.74-2.90 (m, 1H), 2.92-3.10 (m, 1H), 4.59-4.71 (m, 1H), 7.10-7.49 (m 9H). MS ESI m/z: 360.9 $(M + H)^+$. Anal. ($C_{21}H_{26}ClN_2O_2$) C, H, N.

4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)piperidin-4-ol (39). Following method B using 4-chloro-1,1-diphenylbutane (0.82 g, 3.16 mmol), which was synthesized according to literature procedure,[31] and **30** (0.52 g, 2.34 mmol) gave 329 mg (32%) of **39** as a yellow solid. Mp: 110.1-110.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.39-1.79 (m, 5H), 2.00-2.19 (m, 4H), 2.22-2.49 (m, 4H), 2.67-2.83 (m, 2H), 3.89 (t, J = 7.8 Hz, 1H), 7.14-7.43 (m, 14H). MS ESI m/z: 421.2 $(M+H)^+$. Anal. ($C_{27}H_{30}ClNO$) C, H, N.

4-(4-Chlorophenyl)-1-(4-(4-methoxyphenyl)-4-phenylbutyl)piperidin-4-ol (40).

(i) Following method D (step i) starting with 4-methoxybenzophenone (5.75 g, 27.1 mmol) gave 3.18 g (59%) of 4-methoxydiphenylmethane as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 3H), 4.06 (s, 2H), 6.97 (d, *J* = 8.5 Hz, 2H), 7.22-7.41 (m, 7H).

(ii) A solution of potassium-*tert*-butoxide (0.90 g, 8.0 mmol) in THF (20 mL) was added to a solution of *n*-BuLi (5.00 mL, 1.6 M in hexane, 8.0 mmol) in a dry atmosphere at -100 °C. The reaction mixture was stirred for 5 min, and a solution of 4-methoxydiphenylmethane (1.58 g, 7.97 mmol) in THF (15 mL) was added slowly in a period of 15 min. The solution was stirred for 1 h at -95 °C and 1,3-dibromopropane was added in one portion (4.0 mL, 39.4 mmol). The reaction mixture was allowed to warm to room temperature and was stirred for 1.5 h. The solvent was evaporated under reduced pressure, and the residue was diluted with water (25 mL) and extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water (3 × 25 mL) and brine (25 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to afford bromide **50** (R_1 = 4-OCH_3; R_2 = H). The crude product was used without further purification.

(iii) Bromide **50** (R_1 = 4-OCH_3; R_2 = H) was dissolved in CH_3CN, and reacted following method B. Purification by flash chromatography (50% DCM in EtOAc) and recrystallization from EtOAc gave 548 mg (15%) of **40** as white crystals. Mp: 119.7-121.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.47-1.69 (m, 5H), 2.00-2.11 (m, 4H), 2.26-2.43 (m, 4H), 2.69-2.75 (m, 2H), 3.74 (s, 3H), 3.84 (t, *J* = 7.8 Hz, 1H), 6.79 (d, *J* = 8.7 Hz, 2H), 7.11-7.37 (m, 9H), 7.41 (d, *J* = 8.7 Hz, 2H). MS ESI *m*/*z*: 450.4 $(M+H)^+$. Anal. ($C_{28}H_{32}ClNO_2$) C, H, N, Cl.

4-(4-Chlorophenyl)-1-(4-(3,4-dichlorophenyl)-4-phenylbutyl)piperidin-4-ol hydrochloride (41). (i) A solution of $AlCl_3$ (7.36 g, 55.2 mmol) and *tert*-butylamineborane (9.95 g, 111 mmol) in DCM (150 mL) was stirred for 10 min at 0 °C, and a solution of 3,4-dichlorobenzophenone (4.64 g, 18.4 mmol) in DCM (15 mL) was added. The mixture was stirred for 2 h at 0 °C and overnight at room temperature. A cooled solution of 0.1 N HCl (75 mL) at 0 °C was added carefully followed by extraction with EtOAc (100 mL). The combined organic layers were washed with 0.1 N HCl (2 × 75 mL) and brine (100 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by flash chromatography (hexane) to afford 3.43 g (78%) of 3,4-dichloromethane as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 3.93 (s, 2H), 6.99-7.68 (m, 8H).

(ii) A solution of diisopropylamine (1.22 mL, 8.68 mmol) in THF (5 mL) was added to a solution of *n*-BuLi (5.43 mL, 1.6 M in hexane, 8.69 mmol) and stirred for 5 min at -10 °C. The reaction mixture was cooled to -78 °C and a solution of 3,4-dichlorodiphenylmethane (2.07 g, 8.68 mmol) in THF (10 mL) was added slowly in a period of 15 min. The mixture was stirred for 1 h at -10 °C followed by the addition of HMPA (1.5 mL) and 1,3-dibromopropane (4.40 mL, 26.1 mmol) in one portion at -78 °C. The

reaction mixture was allowed to warm to room temperature and stirred for 1 h. Water (25 mL) was added and the solvent was evaporated. The residue was diluted with water (25 mL) and extracted with EtOAc (3 × 25mL). The combined organic layers were washed with water (3 × 25 mL) and brine (25 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by flash chromatography (hexane) to afford 4-bromo-1-(3,4-dichloro-phenyl)-1-phenylbutane **18** (R_1 = 3,4-Cl_2; R_2 = H) as a colourless oil (0.15 g, 5%). ^{1}H NMR ($CDCl_3$): δ 1.65-1.79 (m, 2H), 2.03-2.14 (m, 2H), 3.32 (t, *J* = 6.5 Hz, 2H), 3.78 (t, *J* = 7.5 Hz, 1H), 6.96-7.26 (m, 8H).

(iii) Following method B gave 132 mg (66%) of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. Recrystallization from Et_2O/MeOH gave 124 mg (87%) of **41** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.80-1.86 (m, 4H), 2.01-2.16 (m, 3H), 2.80-3.01 (m, 4H), 3.08-3.46 (m, 4H), 3.87 (t, *J* = 7.8 Hz, 1H), 7.08-7.46 (m, 12H), 12.23 (br s, 1H). MS ESI *m*/*z*: 488.4 $(M+H)^+$. Anal. ($C_{27}H_{29}Cl_4NO \cdot 1.0H_2O$) C, H, N, Cl.

4-(4-Chlorophenyl)-1-(4-(4-chlorophenyl)-4-phenylbutyl)piperidin-4-ol (42). Following method D using 4-chlorobenzophenone gave 256 mg (5% overall yield) of **42** as white crystals. Mp: 126.2-127.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.46-1.53 (m, 2H), 1.64-1.69 (m, 3H), 1.97-2.12 (m, 4H), 2.27-2.43 (m, 4H), 2.68-2.74 (m, 2H), 3.86 (t, *J* = 7.8 Hz, 1H), 7.12-7.43 (m, 13H). MS ESI *m*/*z*: 454.4 $(M+H)^+$. Anal. ($C_{27}H_{29}Cl_2NO$) C, H, N, Cl.

1-(4,4-*bis*-(4-Chlorophenyl)butyl)-4-(4-chlorophenyl)piperidin-4-ol hydrochloride (43). Following method D using 4,4'-dichlorobenzophenone gave 291 mg (5% overall yield) of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. Recrystallization from Et_2O/MeOH gave 302 mg (97%) of **43** as a white solid. Mp: 185.0-185.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.80-1.86 (m, 5H), 2.00-2.11 (m, 2H), 2.70-2.87 (m, 4H), 3.09-3.22 (m, 4H), 3.85 (t, 1H, J= 7.5 Hz), 7.09 (d, *J* = 8.5 Hz, 4H), 7.20-7.26 (m, 6H), 7.39 (d, *J* = 8.6 Hz, 2H), 11.83 (br s, 1H). MS ESI *m*/*z*: 488.4 $(M+H)^+$. Anal. ($C_{27}H_{29}Cl_4NO$) C, H, N, Cl.

4-(4-Chlorophenyl)-1-(4-phenyl-4-*m*-tolylbutyl)piperidin-4-ol hydrochloride (45). Following method D using 3-methylbenzophenone gave 294 mg (15% overall yield) of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. Recrystallization from Et_2O/MeOH gave 299 mg (93%) of **45** as a white solid. Mp: 143-1-144.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.78-1.84 (m, 4H), 2.01-2.13 (m, 3H), 2.29 (s, 3H), 2.80-2.91 (m, 4H), 3.09-3.29 (m, 4H), 3.88 (t, *J* = 7.9 Hz, 1H), 7.00-7.33 (m 11H), 7.41 (d, *J* = 8.5 Hz, 2H), 12.15 (br s, 1H). MS ESI *m*/*z*: 434.4 $(M+H)^+$. Anal. ($C_{28}H_{33}Cl_2NO \cdot 0.17H_2O$) C, H, N, Cl.

4-(4-Chlorophenyl)-1-(4-phenyl-4-*p*-tolylbutyl)piperidin-4-ol hydrochloride (46). Following method D using 4-methylbenzophenone gave 468 mg (19% overall yield) of the free base as a thick oil. This was converted to the hydrochloride salt as described for **3**. One batch was recrystallized

from Et_2O/MeOH to give 304 mg (60%) of **46** as a white solid. Mp: 174.3-176.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.78-1.84 (m, 4H), 1.99-2.12 (m, 2H), 2.26 (s, 3H), 2.69-2.84 (m, 5H), 3.09-3.18 (m, 4H), 3.84 (t, *J* = 7.9 Hz, 1H), 7.07-7.34 (m, 11H), 7.39 (d, *J* = 8.6 Hz, 2H), 11.81 (br s, 1H). MS ESI *m*/*z*: 434.4 $(M+H)^+$. Anal. ($C_{28}H_{32}Cl_2NO \cdot 0.19H_2O$) C, H, N, Cl.

1-(4-Biphenyl-4-y)-4-(4-chlorophenyl)-butyl]-4-(4-chlorophenyl)piperidin-4-ol (47). Following method D using 4-chloro-4'-phenylbenzophenone gave the final compound as a white solid. Recrystallization from EtOAc gave 264 mg (9% overall yield) of **47** as white crystals. Mp: 127.8-128.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.50-1.70 (m, 5H), 2.00-2.13 (m, 4H), 2.30-2.46 (m, 4H), 2.72-2.77 (m, 2H), 3.91 (t, *J* = 7.7 Hz, 1H), 7.16-7.55 (m, 17H). MS ESI *m*/*z*: 530.5 $(M+H)^+$. Anal. ($C_{33}H_{33}Cl_2NO$) C, H, N, Cl.

Pharmacology

Materials. ATP disodium salt, bovine serum albumin, chloroquine diphosphate, and DEAE-dextran (chloride form) were obtained from Sigma. Cell culture media, penicillin, and streptomycin were obtained from Life Technologies, Inc., and fetal calf serum was purchased from Integro B.V. (Dieren, The Netherlands). Myo-[2-^{3}H]Inositol (17 Ci/mmol) was obtained from Perkin-Elmer Life Sciences, and Sephadex G-25 gel filtration columns were purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). The human chemokine CCL5 (regulated on activation, normal T cell expressed and secreted) was obtained from Peprotech (Rocky Hill, NJ).

Cell Culture and Transfection. COS-7 cells were grown at 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM l-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Transfection of the COS-7 cells was performed by DEAE-dextran using 2 μg of DNA of each US28 construct pcDEF3-US28 or empty factor per million cells.[17] The total amount of DNA in transfected cells was maintained constant by addition of the empty vector.

[^{125}I]Chemokine Binding Study. Labeling of CCL5 with [^{125}I] and binding in COS-7 cells were performed as previously described.[20] Briefly, transfected cells were seeded in 24-well plates; 48 h after transfection binding was performed on whole cells for 3 h at 4 °C using 0.3 nM [^{125}I]CCL5 in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% bovine serum albumin) in the presence or absence of varying concentrations of compounds. After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined in the presence of 0.1 μM cold competitor (CCL5).

[^{3}H]Inositol Phoshate Production. Cells were seeded in 24-well plates, and 24 h after transfection they were labeled overnight in inositol-free medium (modified Eagle's medium with Earle's salts) supplemented with 2 mM l-glutamine, l-cysteine, l-leucine, l-methionine, lL-arginine, glucose, 0.2% bovine serum albumin, and 2 μCi/mL *myo*-[2-^{3}H]inositol. Subsequently, the labeling medium was

aspirated, cells were washed for 10 min with Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4) and 20 mM LiCl and incubated for 2 h in the same medium in the absence or presence of varying concentrations of compounds. The incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min of incubation on ice, inositol phosphates were isolated by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

References

1. Britt, W. J.; Alford C. A. Cytomegalovirus. In Fields Virology, 3rd ed.; Fields, B. N., Knipe, D. M., Chanock, R. N., Eds.; Lippincott-Raven: Philadelphia, 1996; pp 2493-2523.
2. Hengel, H.; Weber, C. Driving cells into atherosclerotic lesions- a deleterious role for viral chemokine receptors? *Trends microbiol.* **2000**, *8*, 294-296.
3. Melnick, J. L.; Hu, C.; Burek, J.; Adam, E.; DeBakey, M. E. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* **1994**, *42*, 170-174.
4. Valantine, H. A. The role of viruses in cardiac allograft vasculopathy. *Am. J. Transplant.* **2004**, *4*, 169-177.
5. Zhou, Y. F.; Leon, M. B.; Waclawiw, M. A.; Popma, J. J.; Yu, Z. X.; Finkel, T.; Epstein, S. E. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **1996**, *335*, 624-630.
6. Chee, M. S.; Satchwell, S. C.; Preddie, E.; Weston, K. M.; Barrel, B. G. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature* **1990**, *344*, 774-777.
7. Vink, C.; Smit, M. J.; Leurs, R.; Bruggeman, C. A. The role of cytomegalovirus-encoded homologs of G protein-coupled receptors and chemokines in manipulation of and evasion from the immune system. *J. Clin. Virol.* **2001**, *23*, 43-55.
8. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
9. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.
10. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
11. Billstrom, M. A.; Lehma, L. A.; Scott Worthen, G. Depletion of extracellulair RANTES during human cytomegalovirus infection of endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 163-167.
12. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
13. Randolph-Habecker, J.; Rahill, B.; Torok-Storb, B.; Vieira, J.; Kolattukudy, P. E.; Rovin, B. H.; Sedmak, D. D. The expression of the cytomegalovirus chemokine homolog US28 sequesters biologically active CC chemokines and alters IL-8 production. *Cytokine* **2002**, *29*, 37-46.
14. Kledal, T. N.; Rosenkilde, M. M.; Schwartz, T. W. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett.* **1998**, *441*, 209-214.
15. Streblow, D. N.; Soderberg-Naucler, C.; Vieira, J.; Smith, P.; Wakabayashi, E.; Ruchti, F.; Mattison, K.; Altschuler, Y.; Nelson, J. A. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **1999**, *99*, 511-520.
16. Pleskoff, O.; Treboute, C.; Belot, A.; Heveker, N.; Seman, M.; Alizon, M. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **1997**, *276*, 1874-1878.
17. Casarosa, P; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R. Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
18. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
19. Arvanitakis, L.; Geras-Raaka, E.; Varma, A.; Gershengorn, M. C.; Mesri, E. A. Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature* **1997**, *385*, 347-350.

20. Waldhoer, M.; Kledal, T. N.; Farell, H.; Schwartz, T. W. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. *J. Virol.* **2002**, *76*, 8161-8168.
21. Gruijthuijsen, Y. K.; Casarosa, P.; Kaptein, S. J. F.; Broers, J. L.; Leurs, R.; Bruggeman, C. A.; Smit, M. J.; Vink, C. The rat cytomegalovirus R33-encoded G protein-coupled receptor signals in a constitutive fashion. *J. Virol.* **2002**, *76*, 1328-1338.
22. Bais, C.; Santomasso, B.; Coso, O.; Arvanitakis, L.; Geas-Raaka, E.; Gutkind, J. S.; Asc, A. A.; Cesarman, E.; Gershengorn, M. C. Mesri, E. A. G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature* **1998**, *391*, 86-89.
23. Holst, P. J.; Rosenkilde, M. M.; Manfra, D.; Chen, S. C.; Wiekowski, M. T.; Holst, B.; Cifire, F.; Lipp, M.; Schwartz, T. W. Tumorigenesis indiced by the HHV8-encoded chemokine receptor requires ligand modulation of high constitutive activity. *J. Clin. Invest.* **2001**, *108*, 1789-1796.
24. Hesselgesser, J.; Ng, H. P.; Liang, M.; Zheng, W.; May, K.; Bauman, J. G.; Monahan, S.; Islam, I.; Wei, G. P.; Ghannam, A.; Taub, D. D.; Rosser, M.; Snider, R. M.; Morrissey, M. M.; Perez, H. D.; Horuk, R. Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J. Biol. Chem.* **1998**, *273*, 15687-15692.
25. Ng, H. P.; May, K.; Baumann, J. G.; Ghannan, A.; Islam, I.; Liang, M.; Horuk, R.; Hesselgesser, J.; Snider, R. M.; Perez, H. D.; Morrissey, M. M. Discovery of novel non-peptide CCR1 receptor antagonists. *J. Med. Chem.* **1999**, *42*, 4680-4694.
26. Raveglia, L. F.; Vitali, M.; Artico, M.; Graziani, D.; Hay, D. W. P.; Luttman, M. A.; Mena, R.; Pifferi, G.; Giardina, G. A. M. Investigations of SAR requirements of SR 142801 through an indexed combinatorial library in solution. *Eur. J. Med. Chem.* **1999**, *34*, 825-835.
27. Ismaiel, A. M.; Arruda, K.; Teitler, M.; Glennon, R. A. Ketanserin analogues: the effect of structural modification on 5-HT2 serotonin receptor binding. *J. Med. Chem.* **1995**, *38*, 1196-1202.
28. Moerlein, S. M.; Stöcklin, G. L. Synthesis of high specific activity [^{75}Br] and [^{77}Br]bromperidol and tissue distribution studies in rat. *J. Med. Chem.* **1985**, *28*, 1319-1324.
29. Lau, C. L.; Tardif, S.; Dufresne, C.; Scheigetz, J. Reductive deoxygenation of aryl aldehydes and ketones by *tert*-butylamine-borane and aluminium chloride. *J. Org. Chem.* **1989**, *54*, 491-494.
30. Ono, A.; Suzuki, N.; Kamimura, J. Hydrogenolysis of diaryl and ayl ketonens and carbinols by sodium borohydride and anhydrous aluminium(III). *Synthesis* **1987,** *8*, 736-738.
31. Bunce, R. A.; Sullivan, J. P. A one-step synthesis of 1-halo-ω,ω-diphenylalkanes. *Synthetic Commun.* **1990,** *20*, 865-868.
32. Cordi, A. A.; Snyers, M. P.; Giraud-Mangin, D.; van der Maessen, C.; van Hoeck, J. P.; Beuze, S.; Ellens, E.; Napora, F.; Gillet, C. L.; Gorissen, H.; Calderon, P.; Remacle, M. D.; Janssens de Varebeke, P.; van Dorsser, W.; Roba, J. Synthesis and structure-activity of 4(5)-(2,2-diphenylethyl)imidazoles as new α_2-adrenoreceptor antagonists. *Eur. J. Med. Chem.* **1990**, *25*, 557-568.
33. DeLucca, G. V.; Kim, U. T.; Johnson, C.; Vargo, B. J.; Welch, P. K.; Covington, M.; Davies, P.; Solomon, K. A.; Newton, R. C.; Trainor, G. L.; Decicci, C. P.; Ko, S. S. Discovery and structure-activity relationship of N-(ureidoalkyl)-benzyl-piperidines as potent small molecule CC chemokine receptor-3 (CCR3) antagonists. *J. Med. Chem.* **2002**, *45*, 3794-3804.
34. Subramanyam, B.; Rollema, H.; Woolf, T.; Castagnioli, N. Jr. Identification of a potentially neurotoxic pyridinium metabolite of haloperidol in rats. *Biochem. Biophys. Res. Commun.* **1990**, *166*, 238-244.
35. Wright, A. M.; Bempong, J.; Kirby, M. L.; Barlow, R. L.; Bloomquist, J. R. Effects of haloperidol metabolites on neurotransmitter uptake and release: possible role in neurotoxicity and tardive dyskinesia. *Brain. Res.* **1998**, *788*, 215-222.
36. Harms, A. F.; Hespe, W.; Nauta, W. T.; Rekker, R. F.; Timmerman, H.; de Vries, J. Diphenhydramine derivatives: through manipulation toward design. In *Drug design*, Vol. VI; Ariëns, J. E., Ed.; Academic press: New York, 1975; pp 2-80.
37. Brandsma, L.; Verkruijsse, H. D. Preparative polar organometallic chemistry, Vol. I; Springer-Verlag: Berlin, 1987.

Chapter 3

Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28

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Abstract

G protein-coupled receptors encoded by viruses represent an unexplored class of potential drug targets. In this study we describe the synthesis and pharmacological characterization of the first class of inverse agonists acting on the HCMV-encoded receptor US28. It is shown that replacement of the 4-hydroxy group of lead compound **1** with a methylamine group results in a significant 6-fold increase in affinity. Interestingly, increasing the rigidity of the spacer by the introduction of a double bond leads to a significant increase in binding affinity compared to **1** as well. These novel inverse agonists serve as valuable tools to elucidate the role of constitutive signaling in the pathogenesis of viral infection and may have therapeutic potential as leads for new anti-viral drugs.

Introduction

Chemokines are a group of small (8-14 kDa) soluble proteins that belong to a large family of chemotactic cytokines.[1] They play an important role in the migration and activation of leukocytes in a wide variety of immune-mediated disorders. These proteins are subdivided by structure into four major groups, namely CC, CXC, CX3C and XC chemokines, based on the number and position of conserved cysteine residues in their amino terminus.[2] Chemokines mediate their effects by binding to chemokine receptors, which belong to the family of G protein-coupled receptors. These cell surface proteins are major targets for therapeutic intervention and are targeted by more than 40% of all marketed drugs.[3] Binding of chemokines to their cognate receptors appears promiscuous and redundant, as most chemokine receptors can bind more than one chemokine and most chemokines can activate several chemokine receptor subtypes.[4] However, binding to chemokine receptors is often restricted to a single subclass of chemokines; CC chemokine receptors can only be activated by CC chemokines, while CXC chemokines only bind to CXC chemokine receptors.[2] Two exceptions are the promiscuous chemokine binding protein DARC (Duffy antigen receptor for chemokines), and the human cytomegalovirus (HCMV) encoded receptor US28. DARC binds chemokines of both the CC and CXC subclasses with high affinity,[5] while the viral-encoded receptor US28 binds several CC-chemokines, including CCL2, CCL3, CCL4 and CCL5, as well as the only member of the CX3C chemokine subclass, namely CX3CL1.[6-9]

HCMV is a species specific β-herpesvirus that persists lifelong in the host without any clinical symptomes in immunocompetent individuals. However, the virus can cause severe illness in immunocompromised individuals, like premature neonates, transplant recipients, and human immunodeficiency virus (HIV) infected people.[10,11] After primary infection, the viral genome establishes a lifelong latent infection within the host. The virus has developed several strategies to evade the immune system, such as the expression of genes that mimic host genes that are involved in the immune system.[12-14] One of these viral genes encodes a G protein-coupled receptor, namely US28, with significant homology to mammalian chemokine receptors.[6,8] US28 shows a 30% amino acid sequence homology with the human CCR1 chemokine receptor,[6] suggesting that

HCMV exploits chemokine signaling pathways to interfere with the host immune system through chemokine mimicry.[15] The homology of US28 compared to the CC chemokine receptors is even higher within the *N*-terminus, crucial for chemokine binding, with an amino acid sequence homology of 70% and 52% with the human CCR1 and CCR2 chemokine receptors, respectively.[6]

Currently, the role of US28 is still unknown, but due to its promiscuous chemokine binding profile it is suggested that the receptor acts as a chemokine scavenger by sequestering CC chemokines from the extracellular environment.[16,17] By this means the virus would elude immune surveillance, as chemokines play an important role in the regulation of the immune response. Furthermore, US28 induces the migration of vascular smooth muscle cells upon binding with the chemokines CCL2 and CCL5, which could be exploited by HCMV to disseminate the virus through the human body.[18] This could also provide a possible link between HCMV and the development of vascular diseases, such as arterial restenosis,[19] atherosclerosis,[20] and chronic allograft rejection.[21] Moreover, CCL2 and CCL5 play an important role in the pathogenesis of vascular disease.[22] Like many mammalian chemokine receptors, of which CCR5 and CXCR4 are the primary HIV-1 co-receptors in vivo, US28 can also act as a co-receptor for HIV-1 entry into cells in vitro.[23]

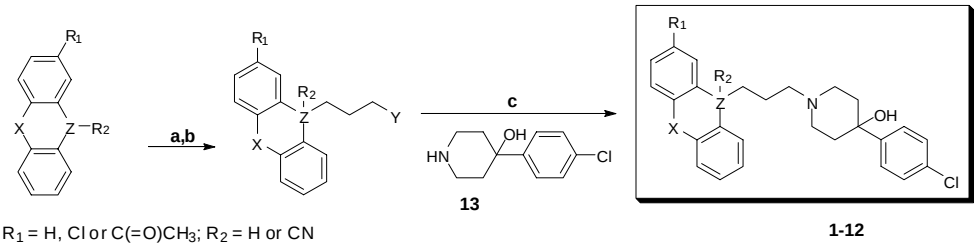
Chemokine receptors have been shown to be involved in the pathophysiology of different diseases.[1,2] The identification of small nonpeptidergic chemokine receptor antagonists that are able to block these receptors proceeds rapidly, with some compounds in clinical trials at the moment. In contrast to mammalian chemokine receptors, we previously showed that US28 signals in a constitutively active manner. The receptor affects different signaling pathways, such as phospholipase C, NF-κB,[24] and the transcription factors nuclear factor of activated T cells (NFAT) and cAMP response element binding protein (CREB).[25-27] The putative role of constitutive activity in viral pathogenesis is not elucidated yet, but it could be a way of the virus to alter the normal homeostasis of a host cell for its own benefit.[24,27] Potent inverse agonists that are able to influence the constitutive signaling of viral-encoded GPCRs could be valuable tools to elucidate the role of constitutive signaling in the pathogenesis of viral infection and may

have therapeutic potential as new anti-viral drugs acting against pathologies caused by HCMV infection. Screening of a variety of GPCR-directed ligands for their ability to modulate the basal signaling of US28 resulted in the identification of the small nonpeptidergic molecule VUF2274 (**1**) as an inverse agonist.[28] This molecule is not only able to block the basal signaling of US28, but also inhibits 60 % of the US28-mediated HIV entry in cells.

Recently, a limited series of analogues of **1** was synthesized to study the very first structure-activity relationships for inverse agonism on US28.[29] To our knowledge, these molecules are currently the only nonpeptidergic inverse agonists acting on a viral-encoded chemokine receptor. In this study, we describe a new series of molecules, in which the rigidity is increased by the introduction of conformationally restrained tricyclic ring systems or rigid fused and nonfused piperidine ring systems. Moreover, we changed the spacer length between the diphenylacetonitrile group and the piperidine moiety and introduced rigidity in this part of the molecule. These novel inverse agonists acting on US28 give us more knowledge about the SAR of this class of compounds.

Chemistry

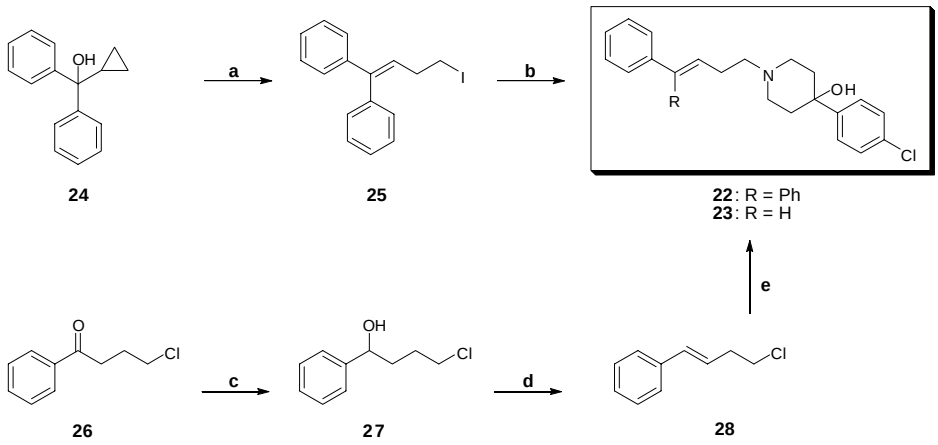
Target compounds **1**-**12** were synthesized via an *N*-alkylation of the appropriate bromide or chloride intermediates with commercially available 4-(4-chlorophenyl)piperidin-4-ol **13** as outlined in Scheme 1.[29-31] Compounds **14**-**21** were synthesized following an analogue synthetic route. Microwave chemistry was used for the *N*-alkylations to shorten the reaction times.



Scheme 1. Synthetic pathway for the synthesis of **1**-**12**. Reagents and conditions: (a) NaH in DMF or di-*n*-butylether; (b) 1-Bromo-3-chloropropane; (c) NaI, Na_2CO_3, CH_3CN, reflux or NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

The tricyclic precursors for compounds **2**-**6** were synthesized following methods previously described in the literature.[31-33] 9*H*-carbazole, 10*H*-phenothiazine, 2-chloro-10*H*-phenothiazine and 1-(10*H*-phenothiazin-2-yl)ethanone were easily deprotonated with NaH at room temperature followed by an alkylation with 1-bromo-3-chloropropane and a reaction with **13** to give target compounds **7** and **10**-**12**.[34,35] However, the deprotonation of 10,11-dihydro-5*H*-dibenzo-[*b*,*f*]azepine and diphenylamine, which were used for the synthesis of **8** and **9**, could not be accomplished under these reaction conditions and was therefore achieved with NaH as a base in di-*n*-butylether at reflux temperature.[36] Cyclohexyl analogue **14** was synthesized starting from cyclohexylphenyl-acetonitrile.[31] 1-Benzyl-4-*tert*-butylbenzene, which was used for the synthesis of **15**, was synthesized from the corresponding benzophenone,[29] and 4-(2-benzothiazolyl)propylamine, an intermediate for the synthesis of **16**, was synthesized using *o*-aminothiophenol and 4-chlorobutyryl chloride.[37]

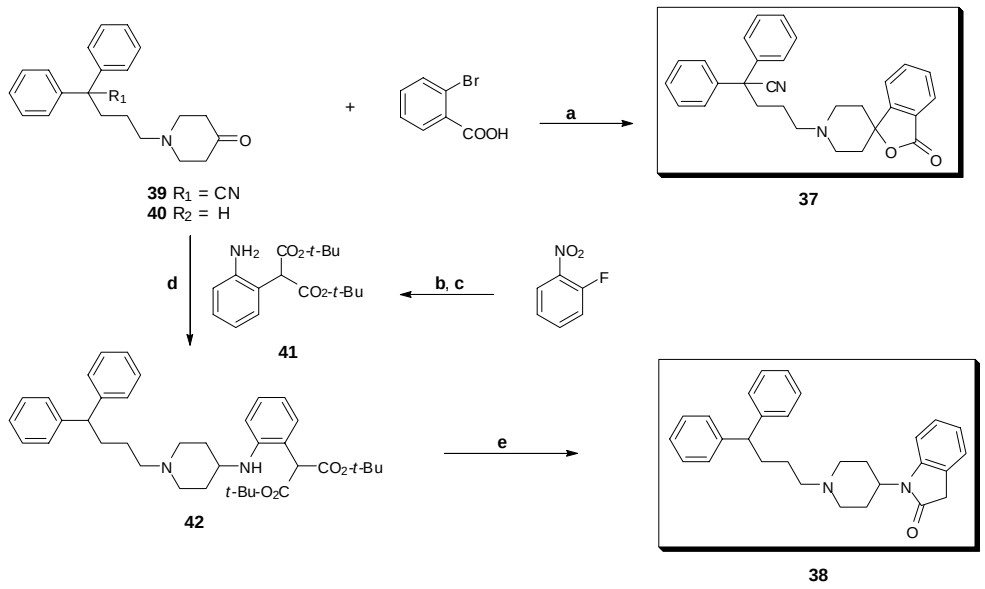
The unsaturated compounds **22** and **23** were synthesized according to the procedure shown in Scheme 2. Cyclopropyldiphenylmethanol **24** was reacted with MgI_2, formed *in situ* from Mg and I_2, to give 4-iodo-1,1-diphenylbut-1-ene **25**, which was reacted with piperidine **13** to give compound **22**.[38,39]



Scheme 2. Synthetic pathway for the synthesis of **22-23**. Reagents and conditions: (a) MgI_2, Et_2O, reflux; (b) **13**, Na_2CO_3, CH_3CN, reflux; (c) $NaHCO_3$, $NaBH_4$, EtOH; (d) HCl, reflux; (e) **13**, NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

A reduction of the carbonyl group of **26** with $NaBH_4$ gave alcohol **27** in a quantitative yield.[40] This alcohol was dehydrated under acidic conditions to give intermediate **28**, which was used for the alkylation of **13** to afford target compound **23**. Compounds **29**-**31** were synthesized following the same method as described in Scheme 1. Reduction of the carbonyl group of **31** with $NaBH_4$ resulted in target compound **32**. The different fused and non-fused piperidine moieties of compounds **33**-**36** were reacted with the appropriate chloride intermediates in a manner similar to that described for target compounds **1**-**12**. The fused and non-fused ring systems of **33**-**34** and **36** were commercially available, and the 6-membered spiropiperidine moiety of **35** was synthesized following a literature procedure.[41] 1-Benzyl-2-methylbenzene, which was used for the synthesis of **34**, was synthesized by the reduction of 2-methylbenzophenone.[29]

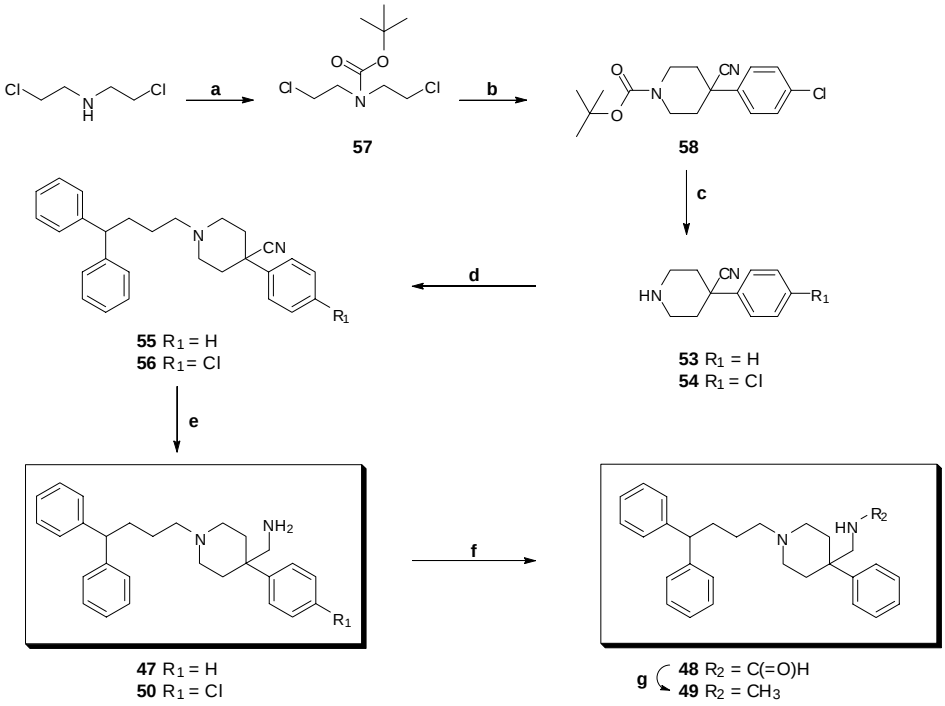
The synthesis of compounds **37** and **38** is outlined in Scheme 3. Compound **37** was synthesized by reacting *o*-bromobenzoic acid with two equivalents of *n*-BuLi,[42] followed by a reaction with ketone **39**.



Scheme 3. Synthetic pathway for the synthesis of **37**-**38**. Reagents and conditions: (a) *n*-BuLi, THF, -78 °C; (b) Di-*t*-butylmalonate, NaH, DMF, 90 °C; (c) Pd/C, H_2, EtOAc; (d) AcOH, $NaBH(OAc)_3$, 1,2-dichloroethane; (e) PTSA, toluene, reflux.

Reductive amination of **40** with di-*t*-butyl 2-(2-aminophenyl)malonate **41**, which was synthesized from the reaction of 2-fluoronitrobenzene with di-*t*-butyl malonate and subsequent reduction of the nitro group, yielded intermediate **42**. The indoline-2-one compound **38** was obtained by an intramolecular cyclisation reaction of **42** by treatment with *p*-toluenesulfonic acid (PTSA).[43] Compounds **43** and **44** were synthesized following a method previously described in the literature.[31] The acetate group of compound **45** was formed by reacting compound **1** with acetylchloride in the presence of triethylamine. Reduction of the methyl ester group of compound **43** with $LiAlH_4$ resulted in alcohol **46**.[31]

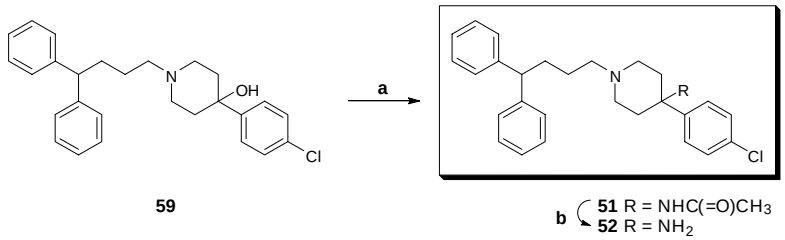
The synthetic route for the preparation of **47**-**52** is outlined in Scheme 4 and 5. Target compounds **47** and **50** were synthesized by a reaction of 4-chloro-1,1-diphenylbutane[44] with **53** or **54** to give intermediates **55** or **56**, followed by a reduction of the nitrile group in the presence of $AlCl_3$ and $LiAlH_4$.



Scheme 4. Synthetic pathway for the synthesis of **47**-**50**. Reagents and conditions: (a) Di-*tert*-butyl dicarbonate, Et_3N, DCM; (b) $NaNH_2$, 2-(4-chlorophenyl)acetonitrile, toluene, 70 °C; (c) EtOH/HCl; (d) 4-Chloro-1,1-diphenylbutane, NaI, Na_2CO_3, CH_3CN, reflux; (e) $AlCl_3$, $LiAlH_4$, THF; (f) Formic acid, microwave (5 min, 200 °C); (g) $LiAlH_4$, THF, reflux.

Compound **53**, which was used for the synthesis of **47**, was commercially available as the hydrochloride salt, but piperidine moiety **54** needed to be synthesized. Thus, 2-(4-chlorophenyl)acetonitrile was deprotonated by $NaNH_2$ and treated with BOC-protected *bis*-(2-chloro-ethyl)amine **57** resulting in intermediate **58**, which was deprotected under acidic conditions to give the desired piperidine moiety **54**.[45] Substituents were easily introduced on the amine group of **47**, so **48** was synthesized in a high yield by reacting **47** with formic acid in the microwave. The monomethyl substituted amine **49** was synthesized by refluxing **48** in THF in the presence of $LiAlH_4$.

The hydroxy group of **59** was converted to an acetamide group in **51** via a Ritter reaction (Scheme 5).[46] In strongly acidic media, a highly electrophilic tertiary carbocation is formed from the tertiary hydroxy group of **59**, and this is followed by an acid-induced nucleophilic addition of the nitrile group of acetonitrile and a hydrolysis resulting in the desired compound **51**. The acetamide group of **51** was hydrolysed under acidic conditions to the corresponding amine group in **52**.[47]



Scheme 5. Synthetic pathway for the synthesis of **51**-**52**. Reagents and conditions: (a) H_2SO_4, CH_3CN; (b) HCl, reflux.

Results and discussion

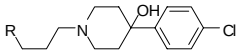
Starting from lead compound **1** we synthesized a novel series of compounds, which were evaluated for their potential to dose-dependently displace [^{125}I]CCL5 binding to US28. The inverse agonistic properties of a selection of compounds was investigated by testing their potential to inhibit the US28-mediated constitutive inositol phosphate production in SVEC4-10 cells.

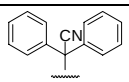
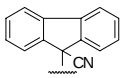
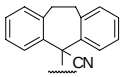
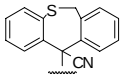
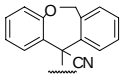
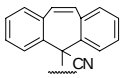
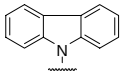
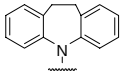
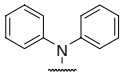
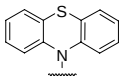
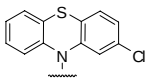
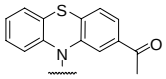
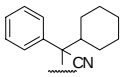
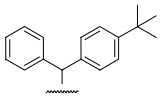
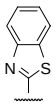
In our previous study[29] it was shown that a piperidine ring was important for activity and therefore this structural motif was maintained. It was also revealed that the nitrile group in the structure was not essential for affinity and efficacy, so this group could be omitted.[29] In this study, we focussed our chemistry program on other parts of the structure, namely on the diphenyl group (Table 1) and the propyl linker (Table 2). Furthermore, we introduced different substitutents at the 4-position of the piperidine ring (Table 3 and 4).

First, more rigidity was introduced by incorporation of the two phenyl rings in different tricyclic moieties (Table 1). The rotation of the two aromatic phenyl rings is restricted by incorporation of an ethylene group between the two phenyl rings in **3**, a bioisosteric thiomethylene or oxomethylene bridge in **4** and **5** or an unsaturated bridge as in **6**. All these changes were tolerated and did not influence the affinity and potency of the compounds. Interestingly, introduction of a nearly planar tricyclic ring system in **2** resulted in a compound with a comparable affinity as well.

The introduction of a nitrogen atom in the tricyclic system as in **7**-**12** could provide an additional position for hydrogen bonding, but this structural modification did not influence the affinity of the compounds. For tricyclic antipsychotics and antidepressants it is known that there is a relationship between the folding of the tricyclic moiety and biological activity. If the sulphur atom of neuroleptic phenothiazine drugs is replaced by an ethylene bridge this results in dibenzazepine derivatives, which have an antidepressant activity.[48] In contrast, replacement of the sulphur atom of phenothiazine analogue **10** into an ethylene bridge, resulting in the tricyclic moiety of dibenzazepine analogue **8**, did not result in any change in affinity or potency on US28. Additionally, introduction of the more rigid and planar carbazole ring system in analogue **7** did not change the affinity for the receptor, as was shown for compound **2** as well. The introduction of a chloro or acetyl group in one of the aromatic rings in phenothiazine analogues **11** and **12** resulted in a decrease in binding affinity, so these substitutions are not preferred in the phenothiazine ring.

Table 1. Chemical structures and pharmacological properties of compounds **1**-**12** and **14**-**16** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



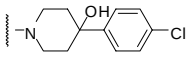
no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
2	10004		6.0 (5.5 - 6.5)	5.7 (4.3 - 7.1)
3	5713		6.6 (4.8 - 8.3)	5.7 (3.0 - 8.5)
4	5727		6.1 (4.6 - 7.6)	5.4 (4.0 - 6.9)
5	10007		7.7 (5.9 - 9.3)	6.2 (3.4 - 8.9)
6	10003		6.0 (4.7 - 7.4)	5.1 (2.5 - 7.8)
7	5932		8.4 (6.2 - 10.7)	4.5 (2.8 - 6.2)
8	5982		6.4 (4.6 - 8.3)	7.1 (3.0 - 11.2)
9	5983		6.9 (3.5 - 10.2)	4.4 (2.4 - 6.3)
10	10005		6.4 (5.2 - 7.6)	6.8 (5.2 - 8.3)
11	10006		10.5 (6.5 - 14.5)	n.d.
12	6902		11.0 (5.5 - 16.6)	10.7 (6.9 - 14.5)
14	5892		7.7 (5.5 - 10.0)	5.2 (3.2 - 7.1)
15	(±)-5937		5.9 (4.6 - 7.2)	7.6 (3.2 - 12.0)
16	5933		18.0 (13.2 - 22.9)	n.d.

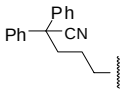
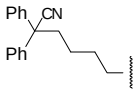
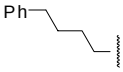
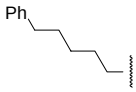
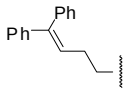
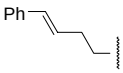
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.d. = not determined.

Previously, we found that introduction of a bulky phenyl substituent at the 4-position of one of the phenyl rings was not allowed to maintain binding affinity.[29] In contrast, the bulky *t*-butyl group in compound **15** is permitted at this position. Interestingly, replacement of the diphenylacetonitrile group by a benzothiazole ring in compound **16** caused a more than 3-fold drop in binding affinity to the receptor.

Furthermore, the importance of the propyl linker between the diphenylacetonitrile group and the piperidine moiety was investigated by the introduction of different structural modifications in this part of the molecule (Table 2).

Table 2. Chemical structures and pharmacological properties of compounds **1** and **17**-**23** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
17	5742		9.2 (7.9 - 10.5)	4.1 (1.5 - 6.6)
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
18	5743		4.6 (3.6 - 5.5)	5.5 (1.9 - 9.1)
19	5745		21.1 (15.8 - 26.3)	n.d.
20	5746		7.8 (6.8 - 8.9)	n.d.
21	5752		6.6 (4.6 - 8.7)	n.d.
22	6869		1.7 (1.1 - 2.3)	4.8 (1.3 – 8.3)
23	(E/Z) 6870		20.0 (19.5 - 20.4)	n.d.

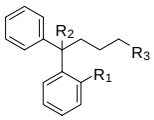
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.d. = not determined.

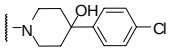
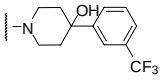
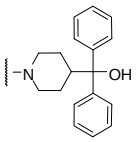
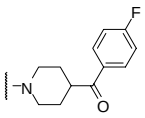
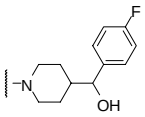
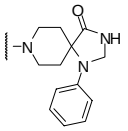
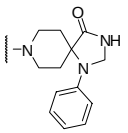
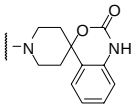
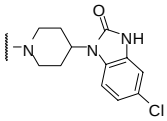
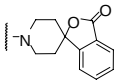
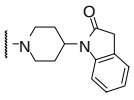
First, we studied the effect of varying the chain length of the linker. Compound **1** has been previously reported as a potent antagonist acting on the human CCR1 chemokine receptor.[34] Interestingly, we recently demonstrated that the SAR of compound **1** and its analogues is completely different on the viral-encoded receptor US28 compared to the human chemokine receptor CCR1.[29] Now, the shortening of the propyl spacer with one methylene group in **17** caused a small decrease in affinity on US28, while the addition of one methylene group in the structure of **1**, resulting in compound **18**, did not change the affinity or efficacy. In contrast, for the human CCR1 chemokine receptor it was shown that shortening of the propyl chain with one methylene group caused a large decrease in K_i value, while addition of one or two methylene groups in the structure of **1**, resulting in, respectively, a butyl and a pentyl chain, did not change the affinity on the CCR1 chemokine receptor.[31] In compounds **19**-**21** one of the phenyl rings was removed and this series of compounds showed a similar trend as demonstrated for compounds **1**, **17** and **18**, namely that removal of one methylene group in **19** resulted in a decrease of binding affinity, while compounds **20** and **21** have binding affinities comparable to compounds **1** and **18**.

Interestingly, the introduction of a more rigid chain in unsaturated analogue **22** resulted in a 3-fold increase in binding affinity compared to lead compound **1**. However, in the functional assay both compounds were equipotent. In our previous study it was shown that removal of a phenyl ring as in **20** resulted in a binding affinity comparable to compound **1**, but this is not true for the more rigid rigid and unsaturated analogues **22** and **23**. Compound **23** has a binding affinity that is more than 10-fold reduced compared to compound **22**.

Next, the influence of the substitution pattern at the 4-position of the piperidine ring was investigated to further define the structure-activity relationships on US28 (Tables 3 and 4). We previously showed that a phenyl ring at this position is important,[29] so this was maintained in the structure. In compounds **33-38** the aromatic ring is incorporated in a heterocyclic system, while in compounds **30**-**32** an additional carbon atom is present between the piperidine ring and the phenyl ring.

Table 3. Chemical structures and pharmacological properties of compounds **1** and **29**-**38** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	VUF	R_1	R_2	R_3	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274	H	CN		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
29	6984	H	CN		5.2 (4.2 - 6.3)	4.3 (2.5 - 6.2)
30	5729	H	CN		4.8 (4.3 - 5.4)	6.1 (2.3 - 10.0)
31	6868	H	H		4.8 (3.9 - 5.6)	n.d.
32	(±)-10010	H	H		6.4 (4.6 - 8.3)	3.0 (2.8 - 3.2)
33	5893	H	CN		3.7 (3.5 - 4.0)	2.0 (1.3 - 2.8)
34	(±)-5997	Me	H		2.6 (1.2 - 3.9)	4.2 (1.1 - 7.4)
35	6967	H	CN		7.6 (6.0 - 9.1)	n.d.
36	6985	H	CN		7.1 (6.3 - 7.9)	n.d.
37	6047	H	CN		11.9 (10.0 - 13.8)	n.d.
38	6048	H	H		5.5 (4.9 - 6.2)	7.3 (2.2 - 12.3)

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.d. = not determined.

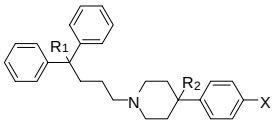
Both the replacement of the 4-chloro atom in the phenyl ring by a 3-trifluoromethyl group in **29** as well as the introduction of a bulky diphenylmethanol group in compound **30** did not influence the potency of the compounds. In compound **31** a 4-fluorophenylmethanone group was introduced at the 4-postion of the piperidine ring and reduction of the carbonyl group resulted in analogue **32**. The introduction of both the 4-fluorophenylmethanone group in compound **31** as well as the 4-fluorophenylmethanol group in analogue **32** did not influence the affinity for US28.

Different fused and non-fused ring systems (heterocyclic substituted piperidine analogues or bicyclic heterocyclic groups) were introduced at the 4-position of the piperidine ring, because these structural motifs appear frequently in CC chemokine receptor antagonists. Additionally, the heterocyclic groups contain different functional groups, which can act as hydrogen bond acceptor or donor groups. Both compounds **33** and **34** contain a spiropiperidine amide group, but compound **34** was not synthesized with a diphenylacetonitrile group as in compound **33**, but with a ortho methyl substituted diphenyl group, because recently it was demonstrated that there was a slight preference for this structural motif.[29] However, in this series of compounds the binding affinities of **33** and **34** were comparable and in the same order as lead compound **1**. Additionally, the introduction of the spiropiperidine moieties in both compounds **35** and **37** were allowed to maintain affinity for US28. The benzimidazolone piperidine moiety of **36** is often seen in ligands acting on various G protein-coupled receptors, such as serotonergic and dopaminergic receptors.[43] Interestingly, the introduction of an indolin-2-one group in compound **38** resulted in a compound with an activity compared to **1**, while the introduction of the benzimidazolone piperidine moiety of **36** made the affinity drop slightly.

Moreover, the 4-hydroxy group was replaced by other substituents (Table 4), because a hydroxy group at this position of a piperidine ring is suggested to be a site of potential metabolic toxicity.[49] The introduction of an ester group in **43**, an acetyl group in **44**, an acetate group in **45** or a hydroxy methyl group in **46** did not result in compounds with a higher affinity. Furthermore, amine analogue **47** was synthesized to investigate the influence of a primary amine group at the 4-

position of the piperidine ring. It was demonstrated earlier that a nitrile group at the 4-position of the piperidine ring was detrimental for both affinity and activity.[29] However, the reduction of this group into a methylamine group of derivative **47** resulted in a 3-fold increase in binding affinity compared to lead compound **1**, while the efficiacy of both compounds was comparable.

Table 4. Chemical structures and pharmacological properties of compounds **1** and **43-52** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	VUF	R_1	R_2	X	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274	CN	OH	Cl	4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
43	5934	CN	$(C=O)OCH_3$	H	8.0 (6.0 - 10.0)	n.d.
44	5984	CN	$(C=O)CH_3$	H	7.9 (6.3 - 9.5)	n.d.
45	5936	CN	$O(C=O)CH_3$	Cl	8.6 (7.4 - 9.8)	n.d.
46	6881	H	CH_2OH	H	7.5 (5.6 - 9.3)	n.d.
47	6046	H	CH_2NH_2	H	1.6 (1.1 - 2.0)	3.1 (1.7 - 4.6)
48	6987	H	$CH_2NHC(=O)H$	H	2.5 (2.0 - 3.0)	n.d.
49	6989	H	CH_2NHCH_3	H	1.3 (1.1 - 1.5)	3.1 (1.2 - 4.9)
50	6966	H	CH_2NH_2	Cl	0.8 (0.7 - 1.0)	3.6 (2.6 - 4.6)
51	6981	H	$NHC(=O)CH_3$	Cl	2.2 (2.0 - 2.5)	4.1 (1.0 - 7.1)
52	6993	H	NH_2	Cl	1.4 (1.3 - 1.4)	5.7 (2.5 - 8.9)

[a] $[^{125}I]$CCL5 displacement. [b] Inhibition of $[^3H]$inositol phosphate production. n.d. = not determined.

From previous studies[28,29] it was known that removal of the *p*-chloro substituent resulted in a decrease in binding affinity. Thus, a chloro atom was introduced at the 4-position of the phenyl ring in compound **50**, which resulted in a further 2-fold increase of binding affinity compared to unsubstituted analogue **47**. The amine group of compound **47** was substituted, resulting in compounds **48** and **49**. Unfortunately, these substitutions did not cause an increase in the binding affinity on US28. Compound **51** was synthesized to investigate the importance of the position of the amine group and this amine was synthesized starting from

compound **52**, in which the amine group is substituted with an acetyl group. Both compounds have an affinity higher than lead compound **1**, but slightly lower than that of our novel lead compound **50**.

Conclusions

In summary, we described the synthesis and structure-activity relationships of inverse agonists acting on the viral-encoded GPCR US28. These molecules are considered as valuable tools to investigate the (patho)physiological role of US28 during viral infection. Replacement of the 4-hydroxy group of lead compound **1** into a methylamine group as in compound **50** resulted in the, to our knowledge, highest affinity inverse agonist acting on US28 currently known. Interestingly, the introduction of a double bond in the propyl linker between the diphenyl group and the piperidine moiety in compound **22** caused a significant increase in binding affinity to US28. Currently, these molecules are being used as tools to further investigate the role of constitutive signaling of US28 in the pathogenesis of viral infection. In the future, potent and selective inverse agonists acting on constitutively active viral GPCRs may have therapeutical potential in the treatment of pathologies caused by viral infections.

Experimental section

General procedures. The solvents were dried according to standard procedures. All reactions were performed under an atmosphere of dry nitrogen. Microwave reactions were performed in a CEM Explorer single mode MW reactor equipped with auto sampler. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. J.T. Baker silica gel was used for flash chromatography. HRMS mass spectra were recorded on a Finnigan MAT 900 mass spectrometer. Melting points were measured on a MPA100 OptiMelt automated melting point system apparatus and were uncorrected. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV-VIS detector set at 254 nm, with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph mass spectrometer. The analyses were performed using the following conditions; condition I: an Alltima(C18)5u column (150 mm x 4.6 mm) with 70% MeOH - 30% H_2O-0.1% formic acid (Method Ia); 60% MeOH - 40% H_2O-0.1% formic acid (Method Ib) or 50% MeOH - 50% H_2O-0.1% formic acid (Method Ic). Flow rate = 1.0 mL/min. Total run time 15 min unless otherwise stated. Condition II: an Alltima(C18)5u column (150 mm x 4.6 mm) with 50% CH_3CN - 50% H_2O-0.1% formic acid (Method IIa); 40% CH_3CN - 60% H_2O-

0.1% formic acid (Method IIb) or 30% CH_3CN - 70% H_2O-0.1% formic acid (Method IIc). Flow rate = 1.0 mL/min. Total run time 20 min. Compounds that were isolated as fumaric acid salts all showed a fumaric acid peak around 2 minutes. Fumaric acid blancs were used to determine the *t*R of fumaric acid. Purities calculated are based on RP HPLC-UV peak surface area of the compounds (disregarding the fumaric acid peak). Reference compounds **1** and **20** have been described previously and were taken from stock.[29] Compounds **3**-**6**, **14**, **17**-**18**, **43** and **46** were synthesized as previously described in the literature[31] and the characterization data confirmed that the desired compounds had been formed. Compounds **15**, **32** and **34** were tested as racemic mixtures.

General method A. 5-(4-Hydroxy-4-(3-(trifluoromethyl)phenyl)piperidin-1-yl)-2,2-diphenylpentanenitrile fumarate (29). 4-(3-(Trifluoromethyl)phenyl)piperidine-4-ol (0.51 g, 2.07 mmol), 5-chloro-2,2-diphenylpentanenitrile[34] (0.54 g, 2.00 mmol), NaI (0.30 g, 2.00 mmol), Na_2CO_3 (0.42 g, 3.96 mmol) and 3 mL CH_3CN were added in a 10 mL microwave vessel and this mixture was reacted during 15 minutes in the microwave at a temperature of 160 °C (settings: ramp time 5 min, hold time 15 min, power 200 watt, pressure 17.2 bar). The solvent was removed in vacuo and the residue was diluted with water (20 mL), followed by an extraction with DCM (3 x 15 mL). The combined organic layers were washed with water (3 x 40 mL) and brine (40 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (0-50% EtOAc in DCM) gave 772 mg (81%) of the free base as an oil. This was dissolved in EtOH and converted to the corresponding fumaric salt by the addition of fumaric acid (0.19 g, 1.6 mmol). The fumaric salt was isolated by evaporation in vacuo and recrystallized from EtOH/Et_2O to give 803 mg (68%) of **29** as a white solid. Mp: 123.7-125.3 °C (dec). ^{1}H NMR (DMSO-d_6): δ 1.47-1.78 (m, 5H), 1.98-2.20 (m, 2H), 2.35-2.91 (m, 8H), 6.57 (s, 2H), 7.33-7.81 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 30.74, 35.31, 36.14, 47.91, 51.18, 55.68, 68.78, 121.49, 121.85, 123.64, 126.44, 127.89, 128.20, 128.69, 128.84, 134.72, 134.91, 139.22, 148.43, 169.59. Anal. RP-HPLC *Ib*: t_R = 6.64 min (purity 100%), *IIb*: t_R = 11.69 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{29}F_3N_2O$: 478.2232; found: 478.2225.

General method B. 5-(4-(Hydroxydiphenylmethyl)piperidin-1-yl)-2,2-diphenylpentane-nitrile (30). A solution of 4-bromo-2,2-diphenylbutanitrile[34] (0.31 g, 1.00 mmol), diphenyl(piperidin-4-yl)methanol (0.36 g, 1.20 mmol), NaI (0.15 g, 1.00 mmol), and Na_2CO_3 (0.22 g, 2.08 mmol) in CH_3CN (30 mL) was refluxed overnight. The solvent was removed in vacuo, the residue was diluted with water (50 mL) and extracted with DCM (3 x 30 mL). The combined organic layers were washed with water (3 x 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (EtOAc) and recrystallization from EtOAc gave 293 mg (59%) of **30** as a white solid. Mp: 77.9-78.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.34-1.69 (m, 5H), 1.85-2.20 (m, 4H) 2.30-2.50 (m, 5H), 2.79-2.99 (m, 2H), 7.11-7.47 (m, 20H). ^{13}C NMR ($CDCl_3$): 22.80, 26.03, 37.25, 43.85,

51.47, 53.71, 57.60, 79.32, 122.22, 125.57, 126.39, 126.69, 127.69, 128.04, 128.71, 129.23, 139.94, 145.70. Anal. RP-HPLC *Ib*: t_R = 9.48 min (purity 98%), *IIa*: t_R = 6.11 min (purity 98%). HRMS (EI) *m/z* calcd for $C_{35}H_{36}N_2O$: 500.2828; found: 500.2817.

General method C. 1-(3-(9*H*-Carbazol-9-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (7). (i) A solution of 9*H*-carbazole (0.84 g, 5.01 mmol) in DMF (20 mL) was cooled to 0 °C and NaH (0.22 g, 5.58 mmol) was added in small portions. After stirring for 1 h at room temperature, the reaction mixture was cooled to 0 °C and 1-bromo-3-chloropropane (0.5 mL, 5.06 mmol) was added. The reaction mixture was allowed to warm to room temperature and stirred overnight. Water (50 mL) was added and the water layer was extracted with EtOAc (3 x 25 mL). The combined organic extracts were washed with water (3 x 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4 and filtered. After evaporation under reduced pressure, the residue was purified by flash chromatography (0-15% DCM in hexane) to give 853 mg (70%) of 9-(3-chloropropyl)-9*H*-carbazole. ^{1}H NMR ($CDCl_3$): δ 2.28-2.49 (m, 2H), 3.51 (t, *J* = 6.0 Hz, 2H), 4.50 (t, *J* = 6.4 Hz, 2H), 7.21-7.31 (m, 2H), 7.40-7.48 (m, 4H), 8.11 (d, *J* = 7.7 Hz, 2H)

(ii) Following method B using 9-(3-chloropropyl)-9*H*-carbazole (0.57 g, 2.34 mmol) gave 727 mg (75%) of **7** as a light yellow solid after recrystallization in EtOAc. Mp: 130.1-131.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.54-1.72 (m, 3H), 2.01-2.14 (m, 4H), 2.28-2.41 (m, 4H), 2.68-2.73 (m, 2H), 4.41 (t, *J* = 6.6 Hz, 2H), 7.17-7.49 (m, 10H), 8.09 (d, *J* = 7.7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 25.91, 38.24, 40.54, 49.17, 55.20, 70.92, 108.62, 118.64, 120.17, 122.67, 125.40, 125.97, 128.27, 132.66, 140.33, 146.60. Anal. RP-HPLC *Ib*: t_R = 9.37 min (purity 100%), *IIb*: t_R = 10.33 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{26}H_{27}ClN_2O$: 418.1812; found: 418.1815.

9-(3-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-propyl)-9*H*-fluorene-9-carbonitrile hydrochloride (2). (i) 9*H*-Fluorene-9-carbonitrile[33] (1.03 g, 5.39 mmol) was dissolved in DMF (25 mL) and NaH (0.22 g, 5.58 mmol) was added in small portions. The reaction was heated to 70 °C and 1-bromo-3-chloropropane (2.75 mL, 27.8 mmol) was added in one portion after 2 h. The reaction mixture was allowed to cool to room temperature and stirred overnight. Water (50 mL) was added and the water layer was extracted with Et_2O (3 x 50 mL). The combined organic extracts were washed with water (3 x 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered and evaporated in vacuo to give 1.06 g of 9-(3-chloropropyl)-9*H*-fluorene-9-carbonitrile as a yellow solid. The crude product was used without further purification.

(ii) Following method A using crude 9-(3-chloropropyl)-9*H*-fluorene-9-carbonitrile (0.49 g, 2.16 mmol) gave 445 mg of the free base as an oil. This was dissolved in Et_2O and dry hydrochloride gas was bubbled through the solution. Isolation of the hydrochloride salt by filtration and recrystallization from MeOH/Et_2O gave 201 mg (17%) of **2** as a white solid. Mp: 156.2-158.1 °C. ^{1}H NMR ($CDCl_3$): δ 1.55-

1.94 (m, 5H), 2.33-2.54 (m, 2H), 2.77-3.18 (m, 6H), 3.26-3.40 (m, 2H), 7.35- 7.60 (m, 8H), 7.66-7.88 (m, 4H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 18.52, 34.88, 36.00, 47.29, 48.64, 56.16, 68.26, 120.45, 120.68, 124.08, 126.17, 128.22, 128.57, 129.67, 132.92, 139.93, 141.57, 145.12. Anal. RP-HPLC *Ib*: t_R = 5.10 min (purity 99%), *IIb*: t_R = 8.39 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{28}H_{27}ClN_2O$: 442.1812; found: 442.1807.

4-(4-Chlorophenyl)-1-(3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)propyl)piperidin-4-ol (8). (i) NaH (0.40 g, 10.03 mmol) was added portion wise to a solution of 10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine (1.95 g, 9.99 mmol) in di-*n*-butylether (30 mL). The reaction mixture was heated, refluxed for 3.5 h, and 1-bromo-3-chloropropane (4.00 mL, 40.5 mmol) was added at 100 °C. The reaction mixture was refluxed overnight, water was added (50 mL) and the water layer was extracted with toluene (3 x 75 mL). The combined organic extracts were washed with water (3 x 100 mL) and brine (100 mL), dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% DCM in hexane) to give 737 mg (27%) of 5-(3-chloropropyl)-10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 1.99-2.16 (m, 2H), 3.18 (s, 4H), 3.57 (t, *J* = 6.4 Hz, 2H), 3.91 (t, *J* = 6.5 Hz, 2H), 6.91-6.99 (m, 2H), 7.08-7.24 (m, 6H).
(ii) Following method A using 5-(3-chloropropyl)-10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepine (0.42 g, 1.55 mmol) gave 494 mg (71%) of **8** as white crystals after recrystallization from EtOAc. Mp: 116.0-117.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.61-1.84 (m, 5H), 1.96-2.11 (m, 2H), 2.26-2.47 (m, 4H), 2.62-2.78 (m, 2H), 3.15 (s, 4H), 3.76 (t, *J* = 6.8 Hz, 2H), 6.85-6.93 (m, 2H), 7.06-7.15 (m, 6H), 7.24-7.41 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 25.74, 32.63, 38.67, 49.33, 49.83, 56.95, 71.40, 120.35, 122.86, 126.48, 126.77, 128.79, 130.22, 133.18, 134.64, 147.17, 148.65. Anal. RP-HPLC *Ib* (total run time 20 min): t_R = 12.71 min (purity 99%), *IIa*: t_R = 4.76 min (purity 100%), *IIb*: t_R = 15.55 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{28}H_{31}ClN_2O$: 446.2125; found: 446.2131.

4-(4-Chlorophenyl)-1-(3-(diphenylamino)propyl)piperidin-4-ol (9). This was synthesized as described for **8** starting with diphenylamine (1.69 g, 10.00 mmol) to give 683 mg (16% over two steps) of **9** as a white solid after recrystallization from EtOAc. Mp: 109.9-111.9 °C. ^{1}H NMR ($CDCl_3$): δ 1.50-1.95 (m, 5H), 2.03-2.22 (m, 2H), 2.32-2.47 (m, 4H), 2.69-2.87 (m, 2H), 3.77 (t, *J* = 7.3 Hz, 2H), 6.88-7.03 (m, 4H), 7.21-7.45 (m, 10H). ^{13}C NMR ($CDCl_3$): δ 25.33, 38.84, 49.86, 50.54, 56.20, 71.47, 121.38, 121.60, 126.49, 128.83, 129.64, 133.23, 136.18, 148.43. Anal. RP-HPLC *Ib*: t_R = 8.62 min (purity 100%), *IIb*: t_R = 11.11 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{26}H_{29}ClN_2O$: 420.1968; found: 420.1970.

1-(3-(10*H*-phenothiazin-10-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (10). Following method C starting with 10*H*-phenothiazine gave 462 mg (34% over two steps) of **10** as a light yellow solid. Mp: 59.0-60.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.51-2.11 (m, 7H), 2.21-2.56 (m, 4H), 2.63-2.89 (m, 2H),

3.93 (t, *J* = 6.7 Hz, 2H), 6.87-7.41 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 25.34, 38.86, 45.61, 49.84, 55.99, 71.28, 115.97, 117.14, 122.90, 125.64, 126.47, 127.63, 127.89, 128.82, 133.26, 145.58. Anal. RP-HPLC *Ia*: t_R = 3.45 min (purity 97%), *Ib* (total run time 20 min): t_R = 13.93 min (purity 98%), *IIb*: t_R = 13.46 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{26}H_{27}ClN_2OS$: 450.1533; found: 450.1539.

1-(3-(2-Chloro-10*H*-phenothiazin-10-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (11). Following method C starting with 2-chloro-10*H*-phenothiazine gave 376 mg (24% over two steps) of **11** as a light yellow solid. Mp: 66.1-66.7 °C. ^{1}H NMR ($CDCl_3$): δ 1.49-1.80 (m, 3H), 1.87-2.18 (m, 4H), 2.30-2.58 (m, 4H), 2.68-2.85 (m, 2H), 3.91 (t, *J* = 6.8 Hz, 2H), 6.84-7.42 (m, 11H). ^{13}C NMR ($CDCl_3$): δ 24.12, 38.33, 45.62, 49.84, 55.71, 71.01, 116.31, 116.33, 122.76, 123.42, 124.08, 125.35, 126.48, 127.87, 127.97, 128.34, 128.83, 133.30, 133.68, 144.77, 146.89. Anal. RP-HPLC *Ib*: t_R = 4.27 min (purity 97%), *IIa*: t_R = 6.45 min (purity 98%). HRMS (EI) *m/z* calcd for $C_{26}H_{26}Cl_2N_2OS$: 484.1143; found: 484.1133.

1-(10-(3-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)propyl)-10*H*-phenothiazin-2-yl)ethanone (12). Following method C starting with 1-(10*H*-phenothiazin-2-yl)ethanone gave 410 mg (22% over two steps) of **12** as a light yellow solid. Mp: 108.9-110.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.39-1.68 (m, 5H), 1.81-2.12 (m, 4H), 2.22-2.81 (m, 4H), 2.50 (s, 3H), 3.96 (t, *J* = 6.7 Hz, 2H), 6.79-6.92 (m, 2H), 7.01-7.42 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 23.71, 26.47, 37.90, 45.11, 49.22, 55.29, 70.70, 113.81, 115.76, 122.75, 123.00, 123.71, 125.92, 126.89, 127.33, 127.54, 128.24, 132.13, 132.65, 136.10, 144.19, 145.33, 197.31. Anal. RP-HPLC *Ib*: t_R = 10.46 min (purity 100%), *IIb*: t_R = 10.90 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{29}ClN_2O_2S$: 492.1638; found: 492.1631.

1-(4-(4-*tert*-Butylphenyl)-4-phenylbutyl)-4-(4-chlorophenyl)piperidin-4-ol (15). This was synthesized following a method previously described[29] starting with 4-*tert*-butylbenzophenone (1.43 g, 6.01 mmol) to give 801 mg (28% over three steps) of **15** as white crystals after recrystallization from hexane/EtOAc. Mp: 104.0–105.3 °C. ^{1}H NMR ($CDCl_3$): δ 1.26 (s, 9H), 1.48-1.72 (m, 5H), 2.02-2.14 (m, 4H), 2.28-2.44 (m, 4H), 2.64-2.79 (m, 2H), 3.85 (t, *J* = 7.8 Hz, 1H), 7.12-7.43 (m, 13H). ^{13}C NMR ($CDCl_3$): δ 27.14, 31.21, 33.15, 34.17, 37.31, 49.08, 50.62, 58.24, 70.45, 125.19, 125.89, 125.99, 127.13, 127.66, 128.30, 128.33, 132.91, 135.46, 141.59, 144.64, 148.71. Anal. RP-HPLC *Ib*: t_R = 5.24 min (purity 100%), *IIa*: t_R = 9.46 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{31}H_{38}ClN_2O$: 475.2642; found: 475.2630.

1-(3-Benzo[*d*]thiazol-2-yl)propyl)-4-(4-chlorophenyl)piperidin-4-ol (16). Following method B using 2-(3-chloropropyl)benzo[*d*]thiazole (0.636 g, 3.00 mmol), which was synthesized according to literature procedure,[37] gave 306 mg (26 %) of **16** as a light yellow solid after recrystallization from EtOAc. Mp: 109.3-110.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.61-1.76 (m, 3H), 2.21-2.39 (m, 4H), 2.50-2.79 (m, 4H), 2.93-3.08 (m, 2H), 3.18 (t, *J* = 7.3 Hz, 2H), 7.24-7.48 (m, 6H), 7.82-7.96 (m,

2H). ^{13}C NMR ($CDCl_3$): δ 25.76, 31.85, 37.48, 49.11, 57.13, 70.51, 121.41, 122.36, 124.68, 125.86, 125.90, 128.34, 132.83, 134.98, 146.01, 152.97, 170.99. Anal. RP-HPLC *Ic*: t_R = 8.42 min (purity 99%), *IIc*: t_R = 10.70 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{21}H_{23}ClN_2OS$: 386.1220; found: 386.1230.

4-(4-Chlorophenyl)-1-(3-phenylpropyl)piperidin-4-ol (19). Following method B using 1-bromo-3-phenylpropane (0.60 g, 3.03 mmol) afforded 729 mg (73%) of **19** as a light yellow solid. Mp: 95.7-96.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.60-1.95 (m, 5H), 2.03-2.21 (m, 2H), 2.36-2.49 (m, 4H), 2.64 (t, *J* = 7.7 Hz, 2H), 2.80-2.86 (m, 2H), 7.16-7.31 (m, 7H), 7.42 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 28.80, 34.12, 38.61, 49.76, 58.44, 71.39, 126.25, 126.49, 128.75, 128.77, 128.83, 133.26, 142.27, 147.06. Anal. RP-HPLC *Ic*: t_R = 6.17 min (purity 100%), *IIc*: t_R = 10.14 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{20}H_{24}ClNO$: 329.1546; found: 329.1549.

4-(4-Chlorophenyl)-1-(5-phenylpentyl)piperidin-4-ol (21). (i) PBr_3 (0.22 mL, 2.34 mmol) was added to a solution of 5-phenyl-1-pentanol (0.82 g, 5.02 mmol) in Et_2O (25 mL) and this was stirred for 24 h at room temperature. Water (50 mL) was added and the solution was basified with K_2CO_3 followed by an extraction with Et_2O (3 x 30 mL). The combined organic extracts were washed with water (100 mL) and brine (100 mL), dried over anhydrous $MgSO_4$ and filtered. Evaporation in vacuo and purification by flash chromatography (hexane) gave 555 mg (49%) of 1-bromo-5-phenylpentane as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 1.36-1.54 (m, 2H), 1.58-1.75 (m, 2H), 1.82-1.98 (m, 2H), 2.61 (t, *J* = 7.5 Hz, 2H), 3.38 (t, *J* = 6.8 Hz, 2H), 7.13-7.38 (m, 5H).

(ii) This was synthesized according to method B using 1-bromo-5-phenylpentane (0.55 g, 2.44 mmol) to give 666 mg (77%) of **21** as white crystals after recrystallization from EtOAc. Mp: 103.4-105.2 °C. ^{1}H NMR ($CDCl_3$): δ 1.30-1.42 (m, 2H), 1.50-1.73 (m, 7H), 2.02-2.19 (m, 2H), 2.34-2.42 (m, 4H), 2.60 (t, *J* = 7.7 Hz, 2H), 2.78-2.84 (m, 2H), 7.15-7.45 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 27.07, 27.66, 31.72, 36.25, 38.66, 49.82, 59.10, 71.41, 126.05, 126.50, 128.65, 128.78, 128.82, 133.22, 142.98, 147.15. Anal. RP-HPLC *Ib*: t_R = 4.57 min (purity 100%), *IIb*: t_R = 6.49 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{22}H_{28}ClNO$: 357.1859; found: 357.1862.

4-(4-Chlorophenyl)-1-(4,4-diphenylbut-3-enyl)piperidin-4-ol (22). Following method B using **25** (1.00 g, 3.00 mmol), which was synthesized according to literature procedure,[38,39] followed by recrystallization from EtOAc/hexane gave 611 mg (49%) of **22** as a white solid. Mp 117.6-118.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.48-1.79 (m, 3H), 1.98-2.20 (m, 2H), 2.29-2.62 (m, 6H), 2.67-2.89 (m, 2H), 6.07 (t, *J* = 7.2 Hz, 1H), 7.15-7.44 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 27.44, 38.27, 49.13, 58.43, 70.88, 125.97, 126.06, 126.83, 126.89, 127.03, 127.08, 127.96, 128.09, 128.25, 129.66, 132.58, 139.86, 142.40, 142.55, 146.80. Anal. RP-HPLC *Ib*: t_R = 10.43 min (purity 100%), *IIb*: t_R = 13.29 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{27}H_{28}ClNO$: 417.1859; found: 417.1845.

4-(4-Chlorophenyl)-1-(4-phenylbut-3-enyl)piperidin-4-ol (23). (i) A solution of HCl (6 mL) and **27** (0.55 g, 2.98 mmol), which was synthesized as previously described,[40] was heated and refluxed for 4.5 h. The reaction mixture was allowed to cool to room temperature, slowly basified with a saturated solution of Na_2CO_3 and the water layer was extracted with EtOAc (3 x 25 mL). The combined organic extracts were washed with water (3 x 50 mL) and brine (50 mL) and dried over anhydrous $MgSO_4$. Evaporation in vacuo and purification by flash chromatography (hexane) gave 99 mg (20%) of **28** as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 2.61-2.72 (m, 2H), 3.61 (t, *J* = 6.9 Hz, 2H), 6.12-6.26 (dt, *J* = 6.9 Hz, 1H), 6.48 (d, *J* = 15.9 Hz, 1H), 7.19-7.38 (m, 5H).

(ii) Following method A using **28** (0.10 g, 0.59 mmol) gave 101 mg (50%) of **23** as white needles after recrystallization from EtOAc. Mp: 136.3-137.2 °C. ^{1}H NMR ($CDCl_3$): δ 1.51-1.82 (m, 3H), 2.02-2.30 (m, 2H), 2.38-2.70 (m, 6H), 2.79-3.00 (m, 2H), 6.12-6.29 (dt, *J* = 6.6 Hz, 1H), 6.44 (d, *J* = 16.0 Hz, 1H), 7.18-7.46 (m, 9H). ^{13}C NMR ($CDCl_3$): δ 30.66, 38.30, 49.21, 58.21, 70.96, 125.83, 125.95, 126.89, 128.27, 128.36, 130.89, 133.01, 137.79. Anal. RP-HPLC *Ic*: t_R = 8.94 min (purity 100%), *IIb*: t_R = 4.79 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{21}H_{24}ClNO$: 341.1546; found: 341.1560.

(1-(4,4-Diphenylbutyl)-piperidin-4-yl)(4-fluorophenyl)methanone (31). This was synthesized according to method B using 4-chloro-1,1-diphenylbutane[44] (1.59 g, 6.50 mmol) and 4-(4-fluorobenzoyl)piperidine toluenesulfonate (2.28 g, 6.00 mmol) to give 1.25 g (50%) of **31** as white crystals after recrystallization from EtOAc. Mp: 88.9-90.6 °C. ^{1}H NMR ($CDCl_3$): δ 1.38-1.53 (m, 2H), 1.69-2.12 (m, 8H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.80-2.99 (m, 2H), 3.06-3.23 (m, 1H), 3.88 (t, *J* = 7.8 Hz, 1H), 6.98-7.34 (m, 12H), 7.80-8.00 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 25.34, 28.59, 33.48, 43.64, 51.17, 53.12, 58.61, 115.36, 115.80, 125.94, 127.68, 128.27, 130.61, 130.79, 144.89, 162.90, 167.96, 200.92. Anal. RP-HPLC *Ib*: t_R = 8.30 min (purity 100%), *IIa*: t_R = 5.13 min (purity 100%), *IIb*: t_R = 16.06 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{30}FNO$: 415.2311; found: 415.2305.

(1-(4,4-Diphenylbutyl)piperidin-4-yl)(4-fluorophenyl)methanol fumarate (32). A solution of **31** (0.16 g, 0.38 mmol) and $NaBH_4$ (0.050 g, 1.32 mmol) in MeOH (20 mL) was stirred overnight at room temperature. The reaction mixture was evaporated in vacuo, water was added (15 mL) and the water layer was extracted with DCM (3 x 10 mL). The combined organic extracts were washed with water (3 x 25 mL) and brine (25 mL), dried over anhydrous $MgSO_4$, filtered and evaporated in vacuo to give 149 mg of the free base as a thick oil. This was dissolved in EtOAc and acidified by the addition of a saturated solution of fumaric acid in Et_2O. The fumaric salt was isolated by filtration and recrystallized from IPA/Et_2O to give 161 mg (79%) of **32** as a white solid. Mp: 77.4-79.3 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.29-1.79 (m, 7H), 1.91-2.40 (m, 5H), 2.61-2.83 (m, 2H), 3.08-3.40 (m, 2H), 3.77 (t, *J* = 7.9 Hz, 1H), 4.12-4.30 (m, 1H), 6.64 (s, 2H), 6.89 (t, *J* = 8.7 Hz, 2H), 7.05-7.24 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 22.03, 25.75, 32.32, 41.46, 50.48, 56.29, 77.08, 114.81, 115.23, 126.25, 127.51, 127.85,

128.00, 128.45, 135.12, 138.56, 143.89, 159.51, 170.43. Anal. RP-HPLC *Ib*: t_R = 6.38 min (purity 100%), *IIb*: t_R = 10.26 min (purity 96%). HRMS (EI) *m/z* calcd for $C_{28}H_{32}FNO$: 417.2468; found: 417.2449.

5-(4-Oxo-1-phenyl-1,3,8-triazaspiro[4.5]decan-8-yl)-2,2-diphenylpentanenitrile (33). Following method B using 1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one (0.83 g, 3.61 mmol) afforded 842 mg (60%) of **33** after recrystallization from $CHCl_3$. Mp: 204.1-206.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.60-1.76 (m, 4H), 2.38-2.68 (m, 10H), 4.70 (s, 2H), 6.45 (s, 1H), 6.80-6.92 (m, 3H), 7.24-7.42 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 23.22, 28.84, 37.34, 49.58, 51.49, 57.38, 59.14, 115.11, 118.75, 122.23, 126.70, 127.71, 128.74, 129.11, 140.07, 142.92, 177.93. Anal. RP-HPLC *Ib*: t_R = 5.41 min (purity 100%), *IIb*: t_R = 8.17 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{30}H_{32}N_4O$: 464.2576; found: 464.2586.

1-Phenyl-8-(4-phenyl-4-*o*-tolylbutyl)-1,3,8-triazaspiro[4.5]decan-4-one (34). Following method A using 1-(4-chloro-1-phenylbutyl)-2-methylbenzene[29] (0.16 g, 0.52 mmol) and 1-phenyl-1,3,8-triaza-spiro[4.5]decan-4-one (0.14 g, 0.62 mmol) gave 188 mg (80%) of **34** as white crystals after recrystallization from hexane/$CHCl_3$. Mp: 184.3-186.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.39-1.83 (m, 4H), 1.93-2.11 (m, 2H), 2.19 (s, 3H), 2.33-2.81 (m, 8H), 4.11 (t, *J* = 7.7 Hz, 1H), 4.80 (s, 2H), 6.61 (br s, 1H), 6.86-6.92 (m, 2H), 7.00-7.38 (m, 12H). ^{13}C NMR ($CDCl_3$): δ 19.78, 29.00, 33.97, 41.28, 46.59, 49.57, 58.24, 58.89, 115.36, 118.89, 125.76, 125.85, 126.41, 128.04, 128.15, 129.12, 130.34, 136.14, 177.56. Anal. RP-HPLC *Ib*: t_R = 8.12 min (purity 100%), *IIb*: t_R = 11.45 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{30}H_{35}N_3O$: 453.2780; found: 453.2795.

5-(2-oxo-1,2-dihydrospiro[benzo[*d*][1,3]oxazine-4,4'-piperidine]-1'-yl)-2,2-diphenylpentanenitrile (35). Following method A using spiro[benzo[*d*][1,3]oxazine-4,4'-piperidin]-2(1*H*)one (0.41 g, 1.62 mmol), which was synthesized according to literature procedure,[41] afforded 416 mg (57%) of **35** as a white solid. Mp: 172.7-174.3 °C. ^{1}H NMR ($CDCl_3$): δ 1.56-1.78 (m, 2H), 1.96-2.21 (m, 4H), 2.34-2.83 (m, 8H), 6.83 (d, *J* = 9.0 Hz, 2H), 6.98-7.56 (m, 12H), 8.89 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 23.65, 35.76, 37.83, 48.59, 52.04, 58.08, 81.91, 114.97, 122.71, 123.66, 124.08, 127.27, 128.28, 129.28, 129.44, 124.71, 140.57, 152.52. Anal. RP-HPLC *Ic*: t_R = 9.84 min (purity 100%), *IIb*: t_R = 4.58 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{29}H_{29}N_3O_2$: 451.2260; found: 451.2263.

5-(4-(5-Chloro-2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazol-1-yl)piperidin-1-yl)-2,2-diphenylpentanenitrile (36). Following method A using 5-chloro-1-(piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one (0.50 g, 2.00 mmol) gave 734 mg (76%) of **36** as a white solid. Mp: 203.1-204.6 °C. ^{1}H NMR (DMSO-d_6): δ 1.35-1.68 (m, 4H), 1.82-2.09 (m, 2H), 2.20-2.59 (m, 6H), 2.77-2.98 (m, 2H), 4.02-4.23 (m, 1H), 6.98-7.04 (m, 2H), 7.20-7.45 (m, 11H), 11.05 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 23.23, 29.02, 37.27, 50.85, 51.49, 52.98, 57.51, 110.21, 120.87, 122.21, 126.66, 126.72, 127.52, 127.77, 128.61, 128.75, 128.82, 139.99, 155.03. Anal. RP-HPLC *Ib*: t_R = 5.81 min (purity

100%), *IIb*: t_R = 8.83 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{29}ClN_4O$: 484.2030; found: 484.2055.

5-(3-oxo-3*H*-spiro[isobenzofuran-1,4'-piperidine]-1'-yl-2,2-diphenylpentanenitrile hydrochloride (37). A solution of *o*-bromobenzoic acid (0.76 g, 3.76 mmol) in THF (20 mL) was cooled to -78 °C and *n*-BuLi (4.7 mL, 1.6 M in hexane, 7.52 mmol) was added slowly in a period of 20 min. The solution was stirred for 2 h at -78 °C and **39** (1.5 g, 4.51 mmol), which was synthesized according to literature procedure,[31] was added slowly in a period of 30 min. The reaction mixture was allowed to warm to room temperature and water (30 mL) was added. The water layer was extracted with Et_2O (5 x 10 mL) and acidified with HCl until a pH of 2. The solution was heated to reflux temperature for 1h, cooled to room temperature and stirred overnight. A 10% NaOH solution was added to a pH of 10, and the water layer was rapidly extracted with $CHCl_3$ (5 x 20 mL). The combined organic extracts were washed with water (3 x 10 mL) and brine (20 mL), dried over anhydrous $MgSO_4$, filtered and evaporated in vacuo to afford 1.01 g of the free base. This was converted to the hydrochloride salt as described for **2** to give 516 mg (31%) of **37** after recrystallization from MeOH/Et_2O. Mp: 129.8-131.5 °C. ^{1}H NMR ($CDCl_3$): δ 1.68-1.92 (m, 4H), 2.48-2.91 (m, 8H), 2.98-3.21 (m, 2H), 7.18-7.60 (m, 12H), 7.66 (d, *J* = 9.0 Hz, 1H), 7.86 (d, *J* = 9.0 Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 21.60, 34.28, 36.80, 49.27, 51.40, 57.08, 82.78, 121.26, 121.94, 125.00, 125.88, 126.56, 127.86, 128.91, 129.63, 134.49, 139.51, 152.25, 169.01. Anal. RP-HPLC *Ic*: t_R = 9.86 min (purity 98%), *IIb*: t_R = 5.64 min (purity 95%). HRMS (EI) *m/z* calcd for $C_{29}H_{28}N_2O_2$: 436.2151; found: 436.2153.

1-(1-(4,4-Diphenylbutyl)piperidin-4-yl)indolin-2-one (38). (i) NaH (1.96 g, 49.00 mmol) was added in 5 min to a solution of di-*t*-butyl malonate (10 mL, 44.61 mmol) in DMF (250 mL) and this was stirred for 15 min. Subsequently, 2-fluoro-nitrobenzene (4.95 mL, 46.83 mmol) was added in one portion and the reaction mixture was stirred for 8 h at 90 °C and two days at room temperature. Water (300 mL) was added and the water layer was extracted with Et_2O (3 x 200 mL). The combined organic extracts were washed with water (4 x 150 mL) and brine (150 mL), dried over Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (20% EtOAc in hexane) to give 3.0 g (20%) of di-*t*-butyl 2-(2-nitrophenyl)malonate as a light brown solid. ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 18H), 5.09 (s, 1H), 7.39-7.78 (m, 3H), 8.01 (d, *J* = 9.1 Hz, 1H).

(ii) This was dissolved in EtOAc (100 mL) and 10% Pd/C (0.35 g) was added. The reaction mixture was hydrogenated with H_2 (5 bar) overnight, filtrated over Hyflo and washed with EtOAc. After evaporated in vacuo, the residue was purified by recrystallization (Et_2O/hexane) to give 915 mg (34%) of **41** as purple crystals. ^{1}H NMR ($CDCl_3$): δ 1.43 (s, 18H), 4.48 (s, 1H), 6.62-6.78 (m, 2H), 7.03-7.19 (m, 2H).

(iii) Both **41** (0.31 g, 1.00 mmol) and **40** (0.31 g, 1.00 mmol), which was synthesized following literature procedures,[29,31] were dissolved in 1,2-dichloroethane (10 mL). Next, molsieves (4Å) and

acetic acid (0.06 mL, 1.00 mmol) were added. The reaction mixture was stirred for 30 min, $NaBH(OAc)_3$ (0.30 g, 1.42 mmol) was added and this was stirred for another 24 h. A saturated solution of $NaHCO_3$ (15 mL) was added and the water layer was extracted with Et_2O (3 x 20 mL). The combined organic extracts were washed with water (3 x 25 mL) and brine (25 mL), dried over $MgSO_4$, filtered and evaporated in vacuo to give 520 mg of **42** as a white solid. The crude product was used without further purification.

(iv) This was dissolved in toluene (20 mL) and *p*-toluene sulphonic acid (0.19 g, 9.99 mmol) was added. The reaction mixture was refluxed for 3 h, quenched with a saturated solution of $NaHCO_3$ (25 mL) and the water layer was extracted with Et_2O (3 x 15 mL). The combined organic extracts were washed with water (3 x 20 mL) and brine (20 mL), dried over anhydrous $MgSO_4$ and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0-50% EtOAc in DCM) and recrystallized from Et_2O to give 112 mg (26%) of **38** as white crystals. Mp: 139.2-140.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.31-1.78 (m, 4H), 1.91-2.19 (m, 4H), 2.22-2.50 (m, 4H), 2.95 (m, 2H), 3.48 (s, 2H), 3.89 (t, *J* = 7.7 Hz, 1H), 4.19-4.35 (m, 1H), 7.02-7.31 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 26.04, 28.45, 31.30, 34.01, 36.25, 50.27, 51.73, 53.69, 58.81, 110.91, 122.19, 124.89, 125.19, 126.52, 127.92, 128.24, 128.83, 144.07, 145.42, 175.25. Anal. RP-HPLC *Ib*: t_R = 5.58 min (purity 99%), *IIb*: t_R = 9.11 min (purity 99%). HRMS (EI) *m/z* calcd for $C_{29}H_{32}N_2O$: 424.2515; found: 424.2501.

5-(4-Acetyl-4-phenylpiperidin-1-yl)-2,2-diphenylpentanenitrile hydrochloride (44).

Following method A using 1-(4-phenylpiperidin-4-yl)ethanone (0.48 g, 2.00 mmol) gave 728 mg of the free base as an oil. This was converted to the hydrochloride salt as described for **2** and recrystallized from MeOH/Et_2O to give 552 mg (54 %) of **44** as a white solid. Mp: 247.1-249.0 °C. ^{1}H NMR ($CDCl_3$): δ 1.75-2.02 (m, 2H), 1.91 (s, 3H), 2.42-3.08 (m, 10H), 3.29-3.51 (m, 2H), 7.11-7.56 (m, 15H), 12.44 (br s, 1H). ^{13}C NMR ($CDCl_3$): δ 20.11, 25.13, 27.23, 28.92, 29.61, 34.87, 48.43, 49.59, 51.17, 121.72, 125.25, 125.53, 126.19, 127.39, 127.85, 139.05, 139.42, 207.72. Anal. RP-HPLC *Ib*: t_R = 4.84 min (purity 100%), *IIb*: t_R = 10.56 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{30}H_{32}N_2O$: 436.2515; found: 436.2525.

4-(4-Chlorophenyl)-1-(4-cyano-4,4-diphenylbutyl)piperidin-4-yl acetate fumarate (45).

Et_3N (0.12 mL, 0.9 mmol) and acetyl chloride (0.02 mL, 0.28 mmol) were added to a solution of **1** (0.13 g, 0.30 mmol) in DCM (5 mL) at 0 °C. The reaction was stirred at 0 °C for 30 min and 1 h at room temperature. Water (10 mL) was added and the water layer was extracted with DCM (3 x 10 mL). The combined organic extracts were washed with water (3 x 15 mL) and brine (15 mL), dried over anhydrous $MgSO_4$, and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0-25% EtOAc in DCM) to afford 89 mg of the free base as a thick oil. This was converted to the fumaric salt as described for **32** and recrystallized from MeOH/Et_2O to give 75 mg

(41%) of **45** as white crystals. Mp: 176.4-177.3 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.42-1.69 (m, 2H), 1.81-2.07 (m, 5H), 2.16-2.56 (m, 8H), 2.65-2.83 (m, 2H), 6.59 (s, 2H), 7.11-7.24 (m, 14H). ^{13}C NMR (DMSO-d_6): δ 21.46, 34.51, 35.57, 48.27, 51.19, 56.18, 64.70, 78.96, 122.21, 126.21, 126.38, 127.68, 127.94, 128.82, 131.37, 133.92, 139.79, 143.16, 166.01, 168.76. Anal. RP-HPLC *Ib*: t_R = 6.42 min (purity 100%), *IIa*: t_R = 5.39 min (purity 100%), *IIb*: t_R = 17.41 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{30}H_{31}ClNO_2$: 486.2074; found: 486.2062.

(1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-yl)methanamine (47). (i) Following method B using 4-chloro-1,1-diphenylbutane[44] (1.22 g, 4.98 mmol) and **53** (0.92 g, 4.15 mmol) gave 1.59 g (81%) of **55** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.36-1.71 (m, 2H), 1.99-2.21 (m, 6H), 2.30-2.56 (m, 4H), 2.88-3.05 (m, 2H), 3.88 (t, *J* = 7.8 Hz, 1H), 7.09-7.51 (m, 15H).

(ii) A suspension of $AlCl_3$ (0.93 g, 6.98 mmol) in THF (10 mL) was added to a suspension of $LiAlH_4$ (0.26 g, 6.85 mmol) in THF (10 mL) at 0 °C and this was stirred for 5 min, followed by the drop wise addition of a solution of **55** (1.36 g, 3.45 mmol) in THF (15 mL). The reaction mixture was allowed to warm to room temperature, stirred overnight, cooled with an ice bath and quenched with a saturated solution of Na_2CO_3 in water until the foaming stopped. Subsequently, the suspension was filtered, and the filtrate was dried over anhydrous $MgSO_4$. Filtration, evaporation in vacuo and purification by flash chromatography (5% Et_3N in EtOAc) afforded 863 mg (63%) of **47** as a white solid. Mp: 228.2-230.2 °C. ^{1}H NMR (MeOH-d_4): δ 1.53-1.72 (m, 2H), 1.98-2.21 (m, 4H), 2.42-2.99 (m, 6H), 3.15 (s, 2H), 3.21-3.40 (m, 2H), 3.94 (t, *J* = 7.9 Hz, 1H), 7.02-7.54 (m, 15H). ^{13}C NMR (DMSO-d_6): δ 22.45, 29.81, 31.80, 47.69, 49.87, 55.33, 64.70, 125.87, 126.70, 126.98, 127.31, 127.48, 128.21, 128.79, 144.60. Anal. RP-HPLC *Ic*: t_R = 3.91 min (purity 100%), *IIc*: t_R = 4.44 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{34}N_2$: 398.2722; found: 398.2741.

***N*-((1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-yl)methyl)formamide hydrochloride (48).** **47** (0.11 g, 0.23 mmol) and formic acid (2 mL) were added in a 10 mL microwave vessel and this was reacted during 5 min in the microwave at a temperature of 200 °C (settings: ramp time 5 min, hold time 5 min, power 200 watt, pressure 17.2 bar). The reaction mixture was quenched with a saturated solution of Na_2CO_3 (2 mL) and the water layer was extracted with DCM (3 x 5 mL). The combined organic extracts were dried over $MgSO_4$ and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% Et_3N in EtOAc) and the free base was converted to the hydrochloride salt as described for **2** to give 100 mg (94%) of **48** as a white solid. Mp: 52.2-52.8 °C. ^{1}H NMR ($CDCl_3$): δ 1.42-1.74 (m, 2H), 1.86-2.42 (m, 8H), 2.50-2.89 (m, 2H), 3.10-3.29 (m, 2H), 3.39 (d, *J* = 7.6 Hz, 2H), 3.87 (t, *J* = 7.8 Hz, 1H), 5.05 (br s, 1H), 7.01-7.61 (m, 15H), 8.03 (s 1H). ^{13}C NMR ($CDCl_3$): δ 24.62, 31.69, 32.24, 33.72, 49.83, 51.45, 58.51, 126.65, 127.42, 128.14, 128.87, 129.00,

129.60, 144.51, 144.96, 161.78. Anal. RP-HPLC *Ib*: t_R = 5.20 min (purity 100%), *IIb*: t_R = 6.08 min (purity 93%). HRMS (EI) *m/z* calcd for $C_{29}H_{34}N_2O$: 426.2671; found: 426.2678.

1-(1-(4,4-Diphenylbutyl)-4-phenylpiperidin-4-ylmethyl)-*N*-methylmethanamine dihydrochloride (49). $LiAlH_4$ (0.75 g, 19.76 mmol) was added to a solution of **48** (0.20 g, 0.47 mmol) in THF (10 mL) at 0 °C. The reaction mixture was refluxed for 6h, quenched with a saturated solution of Na_2CO_3 and the water layer was extracted with DCM (3 x 10 mL). The combined organic extracts were dried over anhydrous $MgSO_4$ and evaporated in vacuo. The residue was purified by flash chromatography (5% Et_3N in EtOAc) to give 150 mg of the free base. This was converted to the corresponding hydrochloride salt as described for **2** to give 104 mg (46%) of **49** as a white solid. Mp: 50.9-52.5 °C. ^{1}H NMR (MeOH-d_4): δ 1.57-1.79 (m, 2H), 1.98-2.27 (m, 4H), 2.59 (s, 3H), 2.60-2.82 (m, 4H), 2.92-3.06 (m, 2H), 3.18-3.30 (m, 2H), 3.40-3.57 (m, 2H), 3.88-4.09 (m, 1H), 7.10-7.33 (m, 10H), 7.36-7.61 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 22.66, 30.45, 35.42, 40.00, 49.34, 51.01, 55.53, 57.59, 61.17, 127.33, 128.08, 128.27, 128.98, 129.03, 130.66, 137.48, 144.28. Anal. RP-HPLC *Ic*: t_R = 3.52 min (purity 100%), *IIc*: t_R = 4.63 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{36}N_2$: 412.2878; found: 412.2861.

(4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)piperidin-4-yl)methanamine dihydrochloride (50). (i) Et_3N (20 mL) was added to a solution of *bis*-(2-chloroethyl)amine hydrochloride (8.17 g, 45.77 mmol) in DCM (75 mL) and this was stirred for 15 min. After the addition of di-*tert*-butyl dicarbonate (10.0 g, 45.8 mmol) in DCM (50 mL), the reaction mixture was stirred for 20 h and quenched with water (200 mL). The water layer was extracted with DCM (4 x 100 mL) and the combined organic extracts were dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (30% hexane in DCM) to give 3.90 g (35%) of **57** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.48 (s, 9H), 3.49-3.70 (m, 8H).

(ii) A suspension of $NaNH_2$ (0.80 g, 20.51 mmol) in toluene (10 mL) was added to a solution of 2-(4-chlorophenyl)acetonitrile in toluene (10 mL) at 0 °C. The reaction mixture was stirred for 30 min at 0 °C and added drop wise to a solution of **57** (2.78 g, 10.01 mmol) in toluene (50 mL) at 0 °C. This was heated to 70 °C, stirred overnight and quenched with water (100 mL). The organic layer was separated and the water layer was extracted with DCM (3 x 50 mL). The combined organic extracts were dried over anhydrous Na_2SO_4 and filtered. Evaporation in vacuo and purification by flash chromatography (DCM) gave 600 mg (19%) of **58** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 9H), 1.72-2.11 (m, 4H), 3.04-3.29 (m, 2H), 4.13-4.35 (m, 2H), 7.31-7.49 (m, 4H).

(iii) This was dissolved in EtOH (30 mL), which was saturated with hydrochloride gas, and the resulting reaction mixture was stirred for 5 min. Evaporation in vacuo gave a quantitative yield of **54** as a white

solid. ^{1}H NMR (DMSO-d_6): δ 2.31-2.44 (m, 4H), 2.99-3.20 (m, 2H), 3.39-3.48 (m, 2H), 7.49-7.62 (m, 4H).

(iv) Following method B using 4-chloro-1,1-diphenylbutane[44] (0.56 g, 2.28 mmol) and **54** (0.49 g, 1.90 mmol) afforded 500 mg (62%) of **56** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.32-1.72 (m, 2H), 1.90-2.08 (m, 6H), 2.23-2.59 (m, 4H), 2.72-3.02 (m, 2H), 3.91 (t, *J* = 7.8 Hz, 1H), 6.99-7.52 (m, 14H).

(v) This was synthesized as described for **47** starting from **56** (0.30 g, 0.47 mmol) to give 130 mg of the free base as an oil. This was converted to the hydrochloride salt as described for **2** to give 100 mg (42%) of **50** as a white solid. Mp: 257.3-258.9 °C (dec). ^{1}H NMR (MeOH-d_4): δ 1.57-1.80 (m, 2H), 2.01-2.29 (m, 6H), 2.56-2.81 (m, 2H), 2.92-3.20 (m, 4H), 3.32-3.54 (m, 2H), 3.82-4.00 (m, 1H), 7.09-7.59 (m, 14H). ^{13}C NMR (DMSO-d_6): δ 21.80, 29.23, 30.48, 31.64, 47.21, 49.80, 54.98, 60.16, 125.92, 127.30, 128.23, 128.80, 131.97, 135.28, 144.46. Anal. RP-HPLC *Ic*: t_R = 6.07 min (purity 100%), *IIc*: t_R = 7.57 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{28}H_{33}ClN_2$: 432.2332; found: 432.2319.

***N*-(4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)-piperidin-4-yl)acetamide (51).** A solution of 95% H_2SO_4 (3.3 mL, 12.4 mmol) was added dropwise to a solution of **59** (1.37 g, 3.26 mmol) in CH_3CN (16 mL), while the temperature was maintained between 25 °C and 30 °C. The reaction mixture was stirred overnight at room temperature, poured into ice and neutralized with a 30% solution of NaOH. Water was added (20 mL) and the water layer was extracted with DCM (3 x 30 mL). The combined organic layers were washed with water (3 x 30 mL) and brine (20 mL), dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (0-50% MeOH in EtOAc) and recrystallized from EtOH/Et_2O to give 655 mg (75%) of **51** as a white solid. Mp: 189.1-190.4 °C. ^{1}H NMR ($CDCl_3$): δ 1.39-1.83 (m, 6H), 1.89-2.50 (m, 6H), 1.98 (s, 3H), 2.67-2.88 (m, 2H), 3.88 (t, *J* = 7.8 Hz, 1H), 5.52 (br s, 1H), 7.13-7.27 (m, 14H). ^{13}C NMR ($CDCl_3$): δ 24.02, 24.52, 33.14, 34.32, 49.21, 50.97, 55.54, 57.82, 126.14, 126.47, 127.30, 127.59, 128.38, 132.51, 143.40, 144.45, 169.58. Anal. RP-HPLC *Ib*: t_R = 5.66 min (purity 100%), *IIb*: t_R = 8.20 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{29}H_{33}ClN_2O$: 460.2281; found: 460.2299.

4-(4-Chlorophenyl)-1-(4,4-diphenylbutyl)piperidin-4-amine fumarate (52). A solution of **51** (0.66 g, 1.42 mmol) in 8% HCl (240 mL) was refluxed for three days. Water was added (40 mL), the water layer was basified with a 20% solution of NaOH and extracted with DCM (3 x 30 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and filtered. After evaporation in vacuo, the residue was purified by flash chromatography (5% MeOH and 1% Et_3N in EtOAc) to give 290 mg of a white solid. This was converted to the fumaric salt as described for **29** to give 110 mg (19%) of **52** as white crystals after recrystallization from MeOH/Et_2O. Mp: 189.9-190.7 °C. ^{1}H NMR (MeOH-d_4): δ 1.57-1.68 (m, 2H), 1.98-2.08 (m, 2H), 2.08-2.18 (m, 2H), 2.13-2.18 (s, 2H), 2.31-2.42 (m, 2H), 1.82-2.95

(m, 4H), 3.15-3.24 (m, 2H), 3.96 (t, *J* = 7.8 Hz, 1H), 7.11-7.19 (m, 2H) 7.23-7.32 (m, 8H) 7.44 (d, *J* = 8.7 Hz, 2H), 7.52 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 23.88, 35.02, 48.17, 50.04, 52.48, 56.42, 125.78, 127.32, 127.79, 127.90, 128.16, 131.43, 134.70, 144.85, 167.23. Anal. RP-HPLC *Ic*: t_R = 5.85 min (purity 100%), *IIc*: t_R = 6.63 min (purity 100%). HRMS (EI) *m/z* calcd for $C_{27}H_{31}ClN_2$: 418.2176; found: 418.2176.

Pharmacology

Transient and stable expression of US28. COS-7 cells were grown as previously described.[50] Transient transfection of COS-7 cells was performed by DEAE-dextran, using 2 μg of US28-pcDEF3 DNA per million cells.[50] SVEC4-10 (ATCC CRL2181) is a cell line derived by SV40 transformation of endothelial cells from the murine axillary lymph node vessels. SVEC4-10 cells were grown as described previously.[50] SVEC4-10 cells were transfected with US28-pTJE8 using 25kDa linear polethylenimine (PEI; Polysciences, Inc.). "Empty" vector was used for mock transfections. Briefly, 10 μg DNA and 50 μg PEI were separately diluted into 250 μl 150 mM NaCl solution. The PEI solution was added to the DNA solution, vortexed and incubated for 15 min at room temperature. One million SVEC4-10 cells were seeded in a 10-cm dish the day before transfection. Culture medium was replaced by 6 mL DMEM and the DNA/PEI solution was added drop wise to the cells. The solution was mixed by gently shaking and the dish was incubated at 37 °C for 5 h. The transfection solution was replaced by culture medium and incubated O/N. SVEC4-10 cells stably expressing US28 were selected in culture medium containing 500 μg/mL neomycin G418. Clones were selected based on US28-mediated constitutive IPx accumulation and chemokine binding analysis.

[^{125}I]CCL5 binding experiments. Radioiodination [^{125}I] of CCL5 (Peprotech, Rocky Hill, NJ) was performed using iodogen (Pierce Chemical Co., Rockford, IL) as described previously.[51] Displacement binding experiments were performed on US28-expressing COS-7 cell membranes. Two days after transfection COS-7 cells were homogenized in ice-cold buffer A (*i.e.* 15 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, and 1 mM EGTA) and centrifuged at 200 x g for 10 min. Supernatant was collected and centrifuged at 48,000 x g for 30 min. The pellet was resuspended in buffer B (*i.e.* 7.5 mM Tris-HCl, pH 7.5, 12,5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, and 250 mM sucrose), aliquoted and stored at -80 °C until use. Cell membranes (approximately 0.2 μg/sample) were incubated with 0.3 nM [^{125}I]CCL5 in binding buffer (*i.e.* 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM NaCl, and 0.2% bovine serum albumin) in the absence or presence of various concentrations of nonpeptidergic ligands at 37 °C for 1 h. Incubations were terminated by filtration through a UniFilter-96 GF/C filter plate (PerkinElmer Life Sciences, Wellesley, MA) presoaked in 0.3% PEI, followed by three rapid washes with ice-cold binding washing buffer (*i.e.* 50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 500 mM

NaCl) using a MicroBeta FilterMate-96 Harvester (PerkinElmer). Non-specific binding was determined on membranes from mock-transfected cells. Radioactivity was quantified with a liquid scintillation using a Wallac MicroBeta TriLux (PerkinElmer).

[^{3}H]inositol phosphate accumulation. SVEC4-10 cells (approximately 4 x 10^5 cells/well) were seeded into poly-L-lysine coated 96-well plates in 200 μl culture medium. After approximately 24h, medium was replaced with 100 μl/well inositol-free Dulbecco's modified Eagle's medium supplemented with 10 μCi/mL *myo*-[2-^{3}H]inositol (PerkinElmer) and the cells were further incubated. The next day, cells were washed and preincubated for 10 min with assay buffer (*i.e.* Dulbecco's modified Eagle's medium containing 10 mM LiCl). Next, cells were incubated in assay buffer in the absence or presence of various concentrations of nonpeptidergic ligands at 37 °C for 2 h. Inositol phosphates were extracted using 10 mM formic acid and quantified using 0.5 mg/well Ysi-RNA-binding SPA beads (Amersham Biosciences), in a white clear bottom 96-well isoplates (PerkinElmer) using a Wallac MicroBeta TriLux, essentially as previously described.[52]

References

1. Baggiolini, M.; Dewald, B.; Moser, B. Human chemokines: an update. *Annu. Rev. Immunol.* **1997**, *15*, 675–705.
2. Murphy, P. M.; Baggiolini, M.; Charo, I. F.; Herbert, C. A.; Horuk, R.; Matsushima, K.; Miller, L. H.; Oppenheim, J. J.; Power, C. A. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev.* **2002**, *52*, 145-176.
3. Brink, C. B.; Harvey, B. H.; Bodenstein, J.; Venter, D. P.; Oliver, D. W. Recent advances in drug action and therapeutics: relevance of novel concepts in G-protein-coupled receptor and signal transduction pharmacology. *Br. J. Clin. Pharmacol.* **2004**, *57*, 373-387.
4. Mantovani, A. The chemokine system: redundancy for robust outputs. *Immunol. Today* **1999**, *20*, 254-257.
5. Neote, K.; Darbonne, W.; Ogez, J.; Horuk, R.; Schall, T. J. Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. *J. Biol. Chem.* **1993**, *268*, 12247-12249.
6. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
7. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.
8. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
9. Kledal, T. N.; Rosenkilde, M. M.; Schwartz, T. W. . Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett.* **1998**, *441*, 209-214.
10. Britt, W. J.; Alford, C. A. In Fields Virology 3rd ed.; Fields, B. N.; Knipe, D. M.; Chanock, R. N., Ed.; Lippincott-Raven: Philadelphia, 1996; pp 2493-2523.
11. Jarvis, M. A.;Nelson, J. A. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Curr. Opin. Microbiol.* **2002**, *5*, 403-407.
12. Davis-Poynter, N. J.; Farrell, H. E. Masters of deception: a review of herpesvirus immune evasion strategies. *Immunol. Cell Biol.* **1996**, *74*, 513-522.
13. Froberg, M. K. Review: CMV escapes! *Ann. Clin. Lab. Sci.* **2004**, *34*, 123-130.
14. Bodaghi, B.; Jones, T. R.; Zipeto, D.; Vita, C.; Sun, L.; Laurent, L.; Arenzana-Seisdedos, F.; Virelizier, J. L.; Michelson, S. Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. *J. Exp. Med.* **1998**, *188*, 855-866.
15. Murphy, P. M. Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat. Immunol.* **2001**, *2*, 116-122.

16. Billstrom, M. A.; Lehma, L. A.; Scott Worthen, G. Depletion of extracellulair RANTES during human cytomegalovirus infection of endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 163-167.
17. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
18. Streblow, D. N.; Soderberg-Naucler, C.; Vieira, J.; Smith, P.; Wakabayashi, E.; Ruchti, F.; Mattison, K.; Altschuler, Y.; Nelson, J. A. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **1999**, *99*, 511-520.
19. Zhou, Y. F.; Leon, M. B.; Waclawiw, M. A.; Popma, J. J.; Yu, Z. X.; Finkel, T.; Epstein, S. E. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **1996**, *335*, 624-630.
20. Melnick, J. L.; Hu, C.; Burek, J.; Adam, E.; DeBakey, M. E. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* **1994**, *42*, 170-174.
21. Valantine, H. A. The role of viruses in cardiac allograft vasculopathy. *Am. J. Transplant.* **2004**, *4*, 169-177.
22. Charo, I. F.; Taubman, M. B. Chemokines in the pathogenesis of vascular disease. *Circ. Res.* **2004**, *95*, 858-866.
23. Pleskoff, O.; Treboute, C.; Belot, A.; Heveker, N.; Seman, M.; Alizon, M. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **1997**, *276*, 1874-1878.
24. Casarosa, P; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R.; Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
25. McLean, K. A.; Holst, P. J.; Martini, L.; Schwartz, T. W.; Rosenkilde, M. M. Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. *Virology* **2004**, *325*, 241-251.
26. Waldhoer, M.; Kledal, T. N.; Farell, H.; Schwartz, T. W. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. *J. Virol.* **2002**, *76*, 8161-8168.
27. Vischer, H. F.; Leurs, R.; Smit, M. J. HCMV-encoded G-protein-coupled receptors as constitutively active modulators of cellular signaling networks. *Trends Pharmacol. Sci.* **2006**, *27*, 56-63.
28. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
29. Hulshof, J. W.; Casarosa, P.; Menge, W. M. P. B.; Kuusisto, L. M.; van der Goot, H.; Smit, M. J.; de Esch, I. J. P.; Leurs, R. Synthesis and structure-Activity relationship of the first nonpeptidergic inverse agonists for the human cytomegalovirus-encoded chemokine receptor US28. *J. Med. Chem.* **2005**, *48*, 6461-6471.
30. Raveglia, L. F.; Vitali, M.; Artico, M.; Graziani, D.; Hay, D. W. P.; Luttman, M. A.; Mena, R.; Pifferi, G.; Giardina, G. A. M. Investigations of SAR requirements of SR 142801 through an indexed combinatorial library in solution. *Eur. J. Med. Chem.* **1999**, *34*, 825-835.
31. Ng, H. P.; May, K.; Baumann, J. G.; Ghannan, A.; Islam, I.; Liang, M.; Horuk, R.; Hesselgesser, J.; Snider, R. M.; Perez, H. D.; Morrissey, M. M. J. Discovery of novel non-peptide CCR1 receptor antagonists. *J. Med. Chem.* **1999**, *42*, 4680-4694.
32. Sindelar, K.; Holubek, J.; Ryska, M.; Svatek, E.; Urban, J.; Protiva, M. 11-(Dimethylaminoalkyl)-6,11-dihydrodibenzo[*b*,*e*]thiepin-11-carbonitriles and some related compounds. *Coll. Czech. Chem. Commun.* **1983**, *48*, 1898-1909.
33. Robarge, M. J.; Husbands, S. M.; Kieltyka, A.; Brodbeck, R.; Thurkauf, A.; Newman, A. H. Design and synthesis of [(2,3-dichlorophenyl)piperazin-1-yl]alkylfluorenylcarboxamides as novel ligands selective for the dopamine D3 receptor subtype. *J. Med. Chem.* **2001**, *44*, 3175-3186.
34. Hesselgesser, J.; Ng, H. P.; Liang, M.; Zheng, W.; May, K.; Bauman, J. G.; Monahan, S.; Islam, I.; Wei, G. P.; Ghannam, A.; Taub, D. D.; Rosser, M.; Snider, R. M.; Morrissey, M. M.; Perez, H. D.; Horuk, R. Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J. Biol. Chem.* **1998**, *273*, 15687-15692.
35. Bright, C.; Brown, T. J.; Cox, P.; Halley, F.; Lockey, P.; McLay, I. M.; Moore, U.; Porter, B.; Williams, R. J. Identification of a non peptidic RANTES antagonist. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 771-774.
36. Andersen, K. E.; Sørensen, J. L.; Lau, J.; Lundt, B. F.; Petersen, H.; Huusfeldt, P. O.; Suzdak, P. D.; Swedberg, M. D. B. Synthesis of novel gamma-aminobutyric acid (GABA) uptake

inhibitors. 5.(1) Preparation and structure-activity studies of tricyclic analogues of known GABA uptake inhibitors. *J. Med. Chem.* **2001**, *44*, 2152-2163.
37. Atwal K. S.; O'Reilly, B. C.; Ruby, E. P.; Turk. C. F.; Aberg, G.; Asaad, M. M.; Bergey, J. L.; Moreland, S.; Powell, J. R. Substituted 1,2,3,4-tetrahydroaminonaphthols: antihypertensive agents, calcium channel blockers, and adrenergic receptor blockers with catecholamine-depleting effects. *J. Med. Chem.* **1987**, 30, 627-635.
38. Saičić R. N.; Čeković, Ž. Cyclopentane ring formation in the cycloaddition reaction of 3-alkenyl radicals to radicophilic olefins. *Tetrahedron* **1990**, *46*, 3627-3640.
39. McCormick, J. P.; Barton, D. L. J. Stereoselective synthesis of homoallylic bromides and iodides. *Chem. Soc. Chem. Commun.* **1975**, *8*, 303-304.
40. Almena, J.; Foubelo, F.; Yus, M. Nitrogen-containing remote functionalised organolithium compounds by reductive opening of five- and six-membered heterocycles. *Tetrahedron* **1996**, *52*, 8545-8564.
41. Berkhout, T. A.; Blaney, F. E.; Bridges, A. M.; Cooper, D. G.; Forbes, I. T.; Gribble, A. D.; Groot, P. H. E.; Hardy, A. Ife, R. J.; Kaur, R.; Moores, K. E. Shillito, H. Willetts, J.; Witherington, J. CCR2: characterization of the antagonist binding site from a combined receptor modeling/mutagenesis approach. *J. Med. Chem.* **2003**, *46*, 4070-4086.
42. Parham, W. E.; Egberg, D. C.; Sayed, Y. A.; Thraikill, R. W.; Keyser, G. E.; Neu, M.; Montgomery, W. C.; Jones, L. D. Spiro piperidines. 1. Synthesis of spiro[isobenzofuran-1(3*H*),4'-piperidin]-3-ones, spiro[isobenzofuran-1(3*H*),4'-piperidines], and spiro[isobenzo-tetrahydrothiophene-1(3*H*),4'-piperidines]. *J. Org. Chem.* **1976**, *41*, 2628-2633.
43. Forbes, I. T. A short and efficient synthesis of *N*-substituted indol-2-ones(oxindoles). *Tetrahedron Lett.* **2001**, *2*, 6943-6945.
44. Bunce, R. A.; Sullivan, J. P. A one-step synthesis of 1-halo-ω,ω-diphenylalkanes. *Synth. Commun.* **1990**, *20*, 865-868.
45. Kwartler, C. E.; Lucas, P. The Preparation of Substituted 4-Aminomethylpiperidines and their Straight Chain Analogs. *J. Am. Chem. Soc.* **1947**, *69*, 2582-2586.
46. Ritter, J. J.; Minieri, P. P. A New Reaction of Nitriles. I. Amides from Alkenes and Mononitriles. *J. Am. Chem. Soc.* **1948**, *70*, 4045-4048.
47. Giardina, G. A. M.; Grugni, M.; Rigolio, R.; Vassallo, M.; Erhard, K.; Farina, C. A reliable and efficient synthesis of SR 142801. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2307-2310.
48. Westkaemper, R. B.; Glennon, R. A. Application of ligand SAR, receptor modeling and receptor mutagenesis to the discovery and development of a new class of 5-HT(2A) ligands. *Curr. Top. Med. Chem.* **2002**, *2*, 575-598.
49. Castagnoli Jr., N.; Rimoldi, J. M.; Bloomquist, J.; Castagnoli, K. P. Potential metabolic bioactivation pathways involving cyclic tertiary amines and azaarenes. *Chem. Res. Toxicol.* **1997**, *10*, 924-940.
50. Casarosa, P.; Waldhoer, M.; LiWang, P. J.; Vischer, H. F.; Kledal, T.; Timmerman, H.; Schwartz, T. W.; Smit, M. J.; Leurs, R. J. CC and CX3C chemokines differentially interact with the N terminus of the human cytomegalovirus-encoded US28 receptor. *J. Biol. Chem.* **2005**, 280, 3275-3285.
51. Gruijthuijsen, Y. K.; Casarosa, P.; Kaptein, S. J. F.; Broers, J. L.; Leurs, R.; Bruggeman, C. A.; Smit, M. J.; Vink, C. The rat cytomegalovirus R33-encoded G protein-coupled receptor signals in a constitutive fashion. *J. Virol.* **2002**, *76*, 1328-1338.
52. Brandish, P. E.; Hill, L. A.; Zheng, W.; Scolnick, E. M. Scintillation proximity assay of inositol phosphates in cell extracts: high-throughput measurement of G-protein-coupled receptor activation. *Anal. Biochem.* **2003**, *313*, 311-318.

Chapter 4

Design of a diverse library of ligands for the viral-encoded GPCR US28

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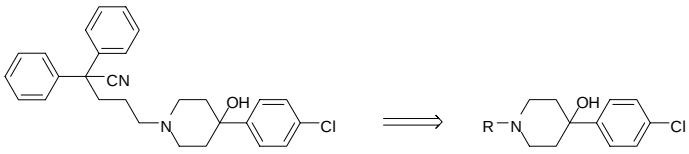
Abstract

Lead compound **1** {5-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile} has recently been identified as the very first inverse agonist acting on the human cytomegalovirus encoded GPCR US28. To identify novel inverse agonists for this receptor that have a significant structural diversity, a focused virtual library around the 4-(4-chlorophenyl)piperidin-4-ol scaffold **2** has been designed. Virtual compounds were generated by the enumeration of commercially available reagents that can react with **2** via standard synthetic procedures, namely carboxylic acids, acid chlorides, sulphonyl chlorides, alkyl halides, aldehydes and ketones. The potential products were processed through different filtering processes to remove compounds with undesirable properties or functional groups. Of the remaining compounds different 1-D and 2-D descriptors, i.e. the logP (o/w), TPSA (2D), the number of single rotatable bonds and the number of H-bond acceptors and donors, were calculated. Based on these descriptors, a diverse subset of 50 compounds was calculated to cover the maximal chemical space of the virtual library. Subsequently, this library was synthesized in a parallel chemistry approach. In the end, 18 compounds could be readily isolated and characterized. Pharmacological evaluation of the synthesized compounds revealed compound **10** as novel inverse agonist acting on US28. All other compounds showed no affinity or efficacy on the receptor.

Introduction

Over the last decades, the G protein-coupled receptor (GPCR) family has emerged as a major target for therapeutic intervention and currently many known marketed drugs are targeted on GPCRs.[1] Chemokine receptors belong to Class A GPCRs, which are characterized by a high homology with rhodopsin. These receptors have been successfully targeted by small molecules and thus constitute a solid database of knowledge that should aid the design of novel ligands for other GPCRs.[2] With the discovery that viruses also encode GPCRs, a new unexplored class of potential drug targets has emerged. Several of these receptors, such as the human cytomegalovirus-encoded (HCMV) US28 or the human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV) receptor ORF74, are suggested to play an important role in the pathogenesis of virus infection.[3] Selective small nonpeptidergic inhibitors for these receptors could serve as new promising therapeutics for innovative anti-viral intervention. However, the constitutively active receptor US28 is currently the only viral-encoded GPCR for which small nonpeptide ligands have been identified.

VUF 2274 (**1**) was the first small nonpeptidergic ligand acting as an inverse agonist on US28 by completely blocking the basal signaling of this receptor.[4] Recently, various analogues of **1** have been synthesized, leading to the very first structure-activity relationships for inverse agonism on a viral-encoded receptor.[5,6] Although many changes were introduced in the structure of **1**, only small improvements in affinity and efficacy were observed. This prompted the question whether more rigorous structural changes where needed to optimize US28 affinity. Therefore, molecular modelling techniques were applied to design a virtual library around the piperidine scaffold (**2**) of lead compound **1**.



1 **2**

Figure 1. Chemical structures of lead compound **1** and piperidine scaffold **2**.

Currently, focussed libraries are considered as a better alternative to the screening of random libraries containing more than 10^6 compounds.[7-9] For many years, screening libraries and corporate compound collections were optimized in terms of size and diversity. Combinatorial chemistry was applied to generate as many compounds as possible and it was believed that the relatively low hit rate from high throughput screening could be overcome by screening more compounds.[10]

It was expected that the combination of HTS and combinatorial chemistry would deliver multiple new promising lead compounds for drug discovery projects, but it has so far not fulfillled all expectations. HTS drug discovery requires large and expensive compound libraries and resulted in high attrition rates in later stages of drug development due to poor physicochemical properties, such as a poor chemical stability in the gut, poor permeability and a poor metabolic and/or elimination profile.[11,12] Thus, it is important that drug-like properties are considered as early as possible and all potential compounds with disadvantageous properties or functional groups should be removed at the earliest possible stage in the drug discovery process.[13-15] Examples of such undesirable molecules are compounds that can interfere with the biological assays, including fluorescent compounds, dyes and promiscuous compounds (compounds of which the activity is not specific for the target).[16] Moreover, chemically toxic or reactive species, such as compounds with electrophilic functional groups, are a potential risk of giving false positives.[16,17]

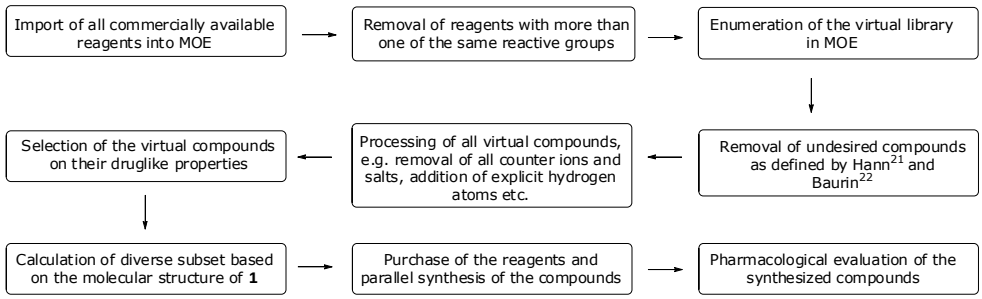
Over the past few years, additional filters have been introduced focusing on more subtle properties, e.g. trying to asses whether or not the ligands are drug-like (suitable to be drugs).[18-21] The first widely used drug-like filter was developed by Lipinski and co-workers,[14] who suggested that poor absorption or permeation are more likely when there are more than 5 hydrogen bond donors (OH and NH), when the molecular weight is over 500, when the calculated octanol/water partition coefficient (CLOGP) is over 5, and when there are more than 10 hydrogen bond acceptors (O and N). This Lipinski rule of five and other drug-like filters can be used as a guide for a proper selection of compounds.[22] This early assessment of a virtual library reduces the size of the library and

improves the chance that any potential hit represents a promising starting point for lead optimization.[23]

In order to find novel structurally diverse inverse agonists for the HCMV-encoded receptor US28 the molecular modelling program MOE (molecular operating environment) was applied to design a small virtual library based on compound **1** by enumeration of commercially available reagents that could potentially react with scaffold **2** via standard synthetic procedures. The virtual library was taken through a filtering process to remove compounds with undesirable features. To ensure maximum coverage of chemical space, but to limit the amount of compounds to be synthesized, a diverse subset based on different descriptors was calculated. This small focussed library was synthesized using parallel chemistry. The synthesized library consists of compounds based on the piperidine scaffold of lead compound **1**. These molecules will provide new important insights about the SAR of this class of compounds.

Methods

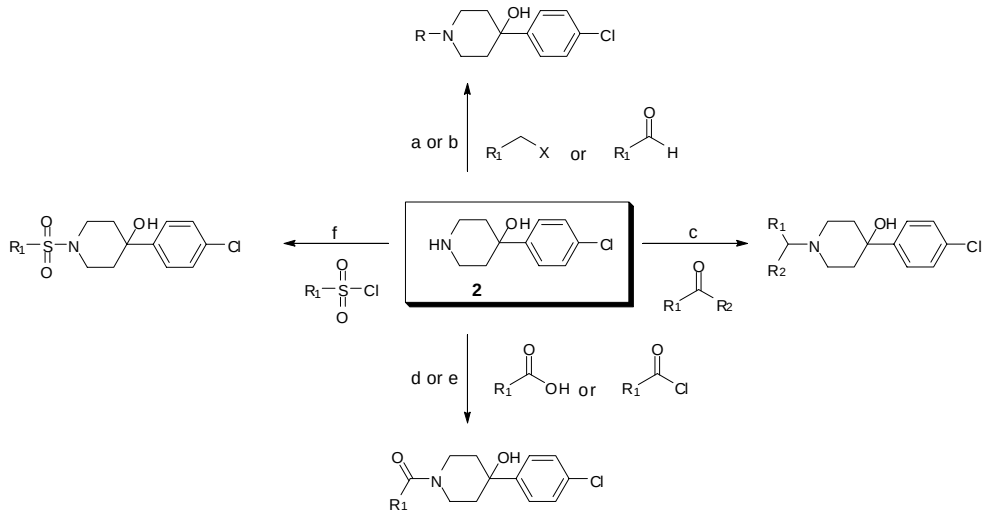
An overview of the approach discussed in this chapter is outlined in Scheme 1. The successive steps will be discussed in detail in the following subsections.



Scheme 1. Flow chart summarizing the successive steps that were followed in this chapter.

Design of the virtual library. The molecular modeling program MOE (Molecular Operating Environment 2004.03) was used to generate a virtual compound library by combinatorial substitution of the commercially available 4-(4-chlorophenyl)piperidin-4-ol (**2**) with reagents selected from the available

chemicals directory (ACD). All commercially available reagents that could potentially react with **2** under reductive amination, acylation or alkylation conditions were selected, namely aldehydes, ketones, alkyl halides, carboxylic acids, acid chlorides and sulfonyl chlorides were considered (Scheme 2).



Scheme 2. Schematic representation of the synthetic procedures applied for the synthesis of the small focussed library. Reagents and conditions: (a) NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C) for alkylchlorides, alkylbromides and alkyliodes (X = Cl, Br or I); (b) AcOH, $NaCNBH_3$, MeOH; (c) Ti(IV)isopropoxide, $NaBH_3CN$, EtOH for ketones; (d) EDCI, HOBT, DIPEA, DCM for carboxylic acids; (e) Et_3N, DCM for acid chlorides; (f) Et_3N, DCM for sulphonyl chlorides.

Suppliers that were taken into account were Acros, Aldrich, Sigma, Lancaster, Fluka and Avocado. Each group of reagents was imported into a separate database and filtered, e.g. reagents that contained more than one of the same reactive group, such as two carboxylic acid groups that can both react with the nitrogen atom of **2**, were removed. The library was generated by virtually reacting piperidine **2** with the reagents from each database under the intended reaction conditions, for example, a reductive amination reaction with the aldehydes or ketones, an alkylation with alkyl halides and an acylation with the carboxylic acids or acid halides. After enumeration of the library, all virtual compounds were combined in one library followed by a second filtering step. The undesired compounds were eliminated by applying a set of structural filters, as defined by Hann[21] and Baurin.[22] Thus, all virtual molecules containing reactive

functional groups, metal atoms or isotopes were removed. Moreover, duplicates, unsuitable lead structures, such as crown ethers or β-lactams, unsuitable natural products and dyes, were expelled.[21,22] The remaining molecules were processed to remove all counter ions and salts, to add all explicit hydrogen atoms, to deprotonate acidic groups and protonate basic groups and to calculate the forcefield partial charges, resulting in a library of 7950 virtual compounds. Next, a drug-like filter based on different 1D and 2D molecular descriptors was applied. All virtual compounds, which did not fulfill the following criteria, were removed: molecular weight ≤ 800, logP (o/w) < 7, number of single rotatable bonds ≤ 10, number of H-bond donors < 5, number of H-bond acceptors < 10, number of halogens < 7 and number of rings < 6. After this process, 5282 entries were left.

Of all these compounds, a selection of 1-D and 2-D descriptors were calculated, i.e. the logP (o/w), TPSA (2D), the number of single rotatable bonds and the number of H-bond acceptors and donors. Lead compound **1** was taken as a starting point and based on the five descriptors, a diverse subset of 50 compounds was calculated. Furthermore, all reagents were passed through a visual inspection and all chemicals were removed that had passed the filtering process, but that were deemed not to be reactive under the chosen reaction conditions, too expensive or not commercially available anymore. To remove as many undesirable compounds as possible, this iterative process was repeated for ten times followed by purchasing all reagents that satisfied the criteria.

Parallel synthesis of the calculated diverse subset. The different synthetic procedures (Scheme 2) were validated and optimized with reagents from our in-house database. The *N*-alkylations of the alkyl halides with piperidine **2** were performed in the microwave in the presence of NaI, Na_2CO_3 and CH_3CN to afford amines **3**-**7** in moderate to high yields.[6] The aldehydes were reacted following a reductive amination procedure utilizing sodium triacetoxyborohydride and acetic acid in 1,2-dichloroethane to give amines **8**-**11**.[24] A reductive amination procedure using titanium(IV) isopropoxide and sodium cyanoborohydride was applied to react the ketones with amine **2**.[25] Unfortunately, this procedure was not successful with the selected ketones and no products were obtained.

Amides **12**-**19** were synthesized using a modified method described by Palani et al.[26] by reacting the carboxylic acids with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 4-hydroxy benzotriazole (HOBT) and diisopropylethylamine (DIPEA) in DCM, followed by the addition of amine **2**. No synthetic procedure for the synthesis of amides by the reaction of piperidine **2** with acid chlorides was applied, because no acid chlorides were present in the selected compounds. Sulphonamide **20** was synthesized in the presence of Et_3N in DCM.

Results and discussion

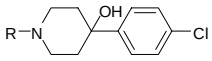
To introduce more rigorous changes into the structure of lead compound **1**, we designed a virtual library around generic structure **2** and a diverse subset was composed based on the calculation of different descriptors. The structural diversity was introduced at the piperidine ring of compound **1**, because previous lead optimization procedures[5,6] had shown that this part of the molecule was important for inverse agonism. However, it was mentioned in Chapter 3 that introduction of a methylamine group attached to the 4-position of the piperidine ring gives a 6-fold increase in binding affinity compared to lead compound **1**, but this substituted piperidine ring was not commercially available and could not be synthesized in large quantities.

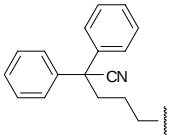
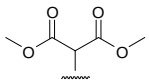
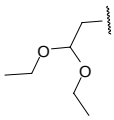
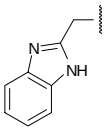
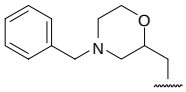
From these 50 reagents, already 8 compounds had to be removed before ordering, because they were not commercially available anymore, too expensive or not reactive under the given reaction conditions (e.g. Michael acceptors that were selected to react with piperidine **2** under reductive aminations conditions). The remaining 42 reagents were purchased and reacted with **2** using the standard synthetic procedures described previously in this chapter. All reactions that did not work were repeated following the same synthetic procedure to confirm that the desired compound could not be formed. The question arises if 42 reagents are enough to obtain satisfactory results. In literature,[27] it was mentioned that general experience over the years has indicated that around 2000 rationally designed compounds have to be synthesized and pharmacologically evaluated to determine good structure-activity relationships. Moreover, it was stated that an interative construction is preferred to acquire a useful final compound. However, this was not possible for us due to time and

cost restraints. Following our approach, 18 compounds were succesfully isolated and pharmacologically evaluated for their potential to dose-dependently displace [^{125}I]CCL5 binding to US28 at a concentration of 10 μM. The inverse agonistic properties of these compounds were investigated by testing their potential to inhibit the US28-mediated constitutive inositol phosphate production in SVEC4-10 cells at the same concentration. For all compounds that showed more than 50% displacement of [^{125}I]CCL5 the IC_{50} and EC_{50} values were obtained.

Table 1 shows the compounds that were synthesized by alkylation of piperidine **2** with the corresponding alkyl halides (compounds **3**-**7**). Unfortunately, none of these compounds showed any affinity or efficacy on US28. Thus, the addition of the two ester moieties in compound **3**, the methyl group in **4** or the ether functionalities in **5** did not result in compounds with any activity on US28.

Table 1. Chemical structures and pharmacological properties of compounds **1** and **3**-**7** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.



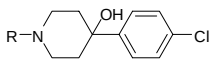
no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
3	10082		n.a.	n.a.
4	10109		n.a.	n.a.
5	10116		n.a.	n.a.
6	10084		n.a.	n.a.
7	10221		n.a.	n.a.

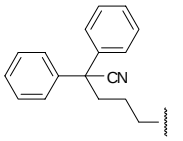
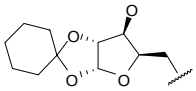
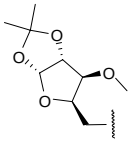
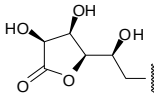
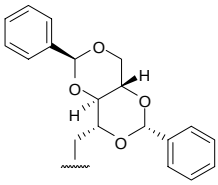
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.a.= not active.

In a previous study, it was shown that the nitrile group of **1** is not essential in the structure and that removal of one of the phenyl rings from the structure resulted in a binding affinity comparable to that of lead compound **1** (see Chapter 2). Moreover, it was shown that the length of the linker between the phenyl ring and piperidine moiety **2** is not of importance, because removal of one methylene group resulted in a slight decrease in binding affinity, while the addition of one methylene group did not change the affinity or efficacy. However, incorporation of the phenyl ring in a benzimidazole ring (**6**) or in a 4-benzyl-morpholine moiety (**7**), resulting in a decrease of conformational freedom, completely abolished activity. A possible explanation of the loss of activity might be that the phenyl rings of both compounds are in the wrong conformation, resulting in a loss of the interaction of the phenyl ring with the receptor.

All selected aldehydes reacted with scaffold **2** under reductive amination conditions (Table 2). The introduction of the sugar moieties, as in compounds **8**-**10**, did not result in compounds with any binding affinity or potency on US28. Interestingly, the introduction of a sugar moiety as linker between two phenyl rings (**11**) did positively influence the activity on US28 and resulted in a 8-fold decrease in binding affinity, while the inverse agonistic properties were only slightly lower.

Table 2. Chemical structures and pharmacological properties of compounds **1** and **8**-**11** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.

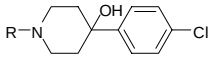


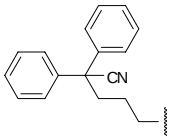
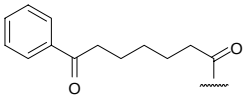
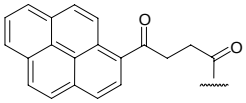
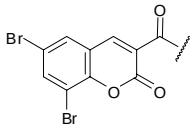
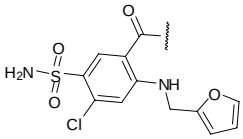
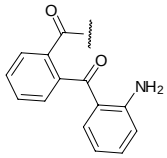
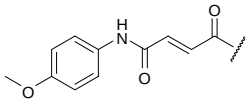
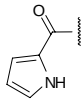
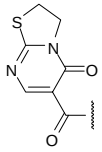
no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
8	10115		n.a.	n.a.
9	10118		n.a.	n.a.
10	10222		n.a.	n.a.
11	10223		42.3 (25.7 – 58.9)	6.2 (3.0 – 9.3)

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.a.= not active.

Table 3 shows the compounds that were successfully synthesized by reacting piperidine **2** with the corresponding carboxylic acids. Despite the high diversity of this selection of compounds, none of them showed any activity on US28. Remarkably, the large lipophilic pyrene group of compound **13** passed all drug-like filters, although this group does not seem to be beneficial in drugs. We did not remove this compound manually, because we wanted to obtain the highest diversity as possible with as only limitation the amount of compounds that could be synthesized due to a lack of reactivity

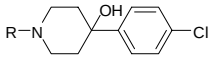
Table 3. Chemical structures and pharmacological properties of compounds **1** and **12**-**19** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.

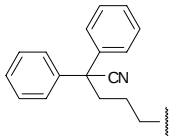
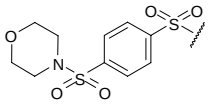


no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
12	10083		n.a.	n.a.
13	10114		n.a.	n.a.
14	10120		n.a.	n.a.
15	10121		n.a.	n.a.
16	10122		n.a.	n.a.
17	10124		n.a.	n.a.
18	10125		n.a.	n.a.
19	10224		n.a.	n.a.

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.a. = not active.

Table 4. Chemical structures and pharmacological properties of compounds **1** and **20** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments, unless otherwise indicated.



no.	VUF	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	2274		4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
20	10119		n.a.	n.a.

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. n.a.= not active.

Only one of the selected reagents had a sulphonyl chloride reactive group, which successfully reacted with piperidine **2** to give sulphonamide **20** (Table 4). Unfortunately, this compound had no affinity nor efficacy on US28 either.

As mentioned earlier in this chapter, none of the selected ketones (11 ketones in totall) reacted with scaffold **2** to the desired products under the chosen reaction conditions, although succesfull reductive amination reactions with titanium(IV) isopropoxide to tertiary amines were previously described.[25,28,29] Due to the lack of reactivity of the selected ketones to react with piperidine **2**, it would have been better if the ketones were not taken into account as reagents.

Conclusions

An approach for the rational design of a focussed virtual library based on the 4-(4-chlorophenyl)piperidin-4-ol scaffold **2** has been developed. The commercially available compounds that could react with **2** under reductive amination, acylation or alkylation conditions were selected. After enumeration of the library and filtering of the virtual compounds, a diverse subset of 50 compounds based on different 1D and 2D descriptors was calculated. Efforts were made to synthesize this selection in a parallel chemistry approach. Eventually, 18 compounds were easily synthesized and characterized. Pharmacological evaluation of the synthesized compounds revealed only compound **11** as as

novel inverse agonists acting on US28. All other variations attached to piperidine ring **2** resulted in compounds with no activity on the receptor.

Experimental section

General procedures. DCM was freshly distilled from lithium aluminium hydride. All reactions were performed under an atmosphere of dry nitrogen. Microwave reactions were performed in a CEM Explorer single mode MW reactor equipped with auto sampler. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. J.T. Baker silica gel was used for flash chromatography. HRMS mass spectra were recorded on a Finnigan MAT 900 mass spectrometer by electron ionization (EI) mass spectrometry or on a Shimadzu IT TOF (ion trap – time-of flight) mass spectrometer by electron spray ionization (ESI) mass spectrometry. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV-VIS detector set at 254 nm, with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph mass spectrometer. The analyses were performed using two of the following conditions; condition I: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 70% MeOH - 30% H_2O-0.1% formic acid (Method Ia); 60% MeOH - 40% H_2O-0.1% formic acid (Method Ib) or 50% MeOH - 50% H_2O-0.1% formic acid (Method Ic). Flow rate = 1.0 mL/min. Total run time 15 min unless otherwise stated. Condition II: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 50% CH_3CN - 50% H_2O-0.1% formic acid (Method IIa); 40% CH_3CN - 60% H_2O-0.1% formic acid (Method IIb), 30% CH_3CN - 70% H_2O-0.1% formic acid (Method IIc) or 20% CH_3CN - 80% H_2O-0.1% formic acid (Method IId). Flow rate = 1.0 mL/min. Total run time 20 min unless otherwise stated. Condition III: a Xbridge(C18)5um column (100 mm x 4.9 mm) with solvent A, 5% CH_3CN - 95% H_2O with 0.1% formic acid; solvent B, 90% CH_3CN - 10% H_2O with 0.1% formic acid; flow rate = 2.0 mL/min; start: 100% A, linear gradient time 10 min, then 5 min at 100% B, then 15 min at 100% A. Total run time 30 min (method IIIa) or start: 100% A, lineair gradient time 5 min, then 5 min at 100% B, then 10 min at 100% A. Total run time 20 min (method IIIb). Condition IV: a Xbridge(C18)5um column (100 mm x 4.9 mm) with solvent A, 5% CH_3CN - 95% H_2O with 10% NH_4HCO_3/NH_4OH buffer pH 8; solvent B, 90% CH_3CN - 5% H_2O with 10% NH_4HCO_3/NH_4OH buffer pH 8; flow rate = 2.0 mL/min; start: 100% A, linear gradient time 10 min, then 5 min at 100% B, then 15 min at 100% A. Total run time 30 min (method IVa or start: 100% A, lineair gradient time 5 min, then 5 min at 100% B, then 10 min at 100% A. Total run time 20 min (method IVb). Compounds that were isolated as fumaric acid salts all showed an extra peak around two minutes. Fumaric acid blancs were used to determine the *t*R of fumaric acid. Purities calculated are based on RP HPLC-UV peak surface area of the compounds (disregarding the fumaric acid peak). Reference compound **1** has been described previously and was taken from stock.[5]

General method A for the synthesis of target ccompounds 3-7. Dimethyl 2-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)malonate (3). 4-(4-Chlorophenyl)-4-piperidinol **2** (0.21 g, 1.00 mmol), dimethyl 2-bromomalonate (0.21 g, 1.00 mmol), NaI (0.15 g, 1.00 mmol), Na_2CO_3 (0.21 g, 2.00 mmol) and 3 mL CH_3CN were added in a microwave vessel and this was reacted during 15 minutes in the microwave at a temperature of 160 °C (settings: ramp time 5 min, hold time 15 min, power 200 watt, pressure 17.2 bar). The solvent was removed in vacuo and the residue was diluted with water (20 mL), followed by an extraction with DCM (3 x 15 mL). The combined organic layers were washed with water (3 x 30 mL) and brine (30 mL), dried over anhydrous $NaSO_4$, filtered, and evaporated in vacuo to give 132 mg (39%) of **3** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.61-1.82 (m, 2H), 2.08-2.31 (m, 2H), 2.82-2.98 (m, 4H), 3.81 (s, 6H), 4.13 (s, 1H), 7.22-7.50 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 38.48, 46.31, 52.27, 70.62, 70.82, 125.89, 128.28, 132.70, 146.51, 167.37. Anal. RP-HPLC *Id*: t_R = 10.45 min (purity 100%), *IV*: t_R = 11.92 min (purity 96%). HRMS (EI) m/z calcd for $C_{16}H_{20}ClNO_5$: 341.1030; found: 341.1015.

4-(4-Chlorophenyl)-1-methylpiperidin-4-ol (4) Yield: 197 mg (87%) of **4** as white crystals. ^{1}H NMR ($CDCl_3$): δ 1.62-1.81 (m, 3H), 2.08-2.27 (m, 2H) 2.31-2.52 (m, 5H), 2.69-2.81 (m, 2H), 7.23-7.44 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 38.19, 46.09, 51.27, 70.03, 125.96, 128.22, 132.53, 146.94. Anal. RP-HPLC *Ig*: t_R = 11.45 min (purity 100%), *IId*: t_R = 4.06 min (purity 100%). HRMS (EI) m/z calcd for $C_{12}H_{16}ClNO$: 225.0920; found: 225.0928.

4-(4-Chlorophenyl)-1-(2,2-diethoxyethyl)piperidin-4-ol hydrochloride (5). Yield: 257 mg (71%). ^{1}H NMR ($CDCl_3$): δ 1.05-1.39 (m, 6H), 1.73-1.98 (m, 2H), 2.63-3.13 (m, 4H), 3.26-3.89 (m, 8H), 5.19-5.34 (m, 1H), 7.11-7.54 (m, 4H). ^{13}C NMR (MeOH-d_4): δ 15.60, 36.42, 51.83, 59.68, 65.01, 69.00, 98.79, 127.50, 129.51, 134.24, 147.09. Anal. RP-HPLC *IIIb*: t_R= 9.02 min (purity 94%), *IVb*: t_R = 9.81 min (purity 96%). HRMS (ESI) m/z calcd for $C_{17}H_{27}Cl_2NO_3$: 363.1368; found: 363.1360.

1-((1*H*-benzo[*d*]imidazol-2-yl)methyl)-4-(4-chlorophenyl)piperidin-4-ol (6). Yield: 161 mg (47%). ^{1}H NMR (MeOH-d_4): δ 1.95-2.21 (m, 2H), 2.46-2.75 (m, 2H), 3.63-3.92 (m, 4H), 5.05 (s, 2H), 7.34-8.07 (m, 8H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 34.50, 48.88, 59.41, 67.08, 114.35, 125.42, 125.84, 127.55, 131.83, 132.28, 141.23, 145.32. Anal. RP-HPLC *Id*: t_R = 6.81 min (purity 100%), *IIIa*: t_R = 12.45 min (purity 99%), *IVa*: t_R = 11.62 min (purity 96%). HRMS (ESI) m/z calcd for $C_{19}H_{20}ClN_3O$: 341.1295; found: 341.1289.

1-((4-Benzylmorpholin-2-yl)methyl)-4-(4-chlorophenyl)piperidin-4-ol dihydrochloride (7). Yield: 91 mg (19%). ^{1}H NMR (MeOH-d_4): δ 1.87-1.99 (m, 2H), 2.31-2.48 (m, 2H), 2.97-3.08 (m, 1H), 3.19-3.70 (m, 10H), 3.98-4.06 (m, 1H), 4.15-4.24 (m, 1H), 4.33-4.54 (m, 3H), 7.36 (d, *J* = 8.6 Hz, 2H), 7.42-7.65 (m, 7H). ^{13}C NMR (MeOH-d_4): δ 36.45, 52.06, 52.32, 53.22, 58.82, 62.36, 64.74, 68.93, 69.59, 127.45, 129.53, 130.47, 131.52, 132.69, 134.38, 147.21. Anal. RP-HPLC *If*: t_R = 5.44

min (purity 100%), *IIIa*: t_R = 9.55 min (purity 96%), *IVa*: t_R = 12.86 min (purity 100%). HRMS (ESI) m/z calcd for $C_{23}H_{29}ClN_2O_2$: 400.1918; found: 400.1918.

General method B for the synthesis of target compounds 8-11 . 4-(4-Chlorophenyl)-1-(((3a'*R*,5'*R*,6'*S*,6a'*R*)-6'-hydroxytetrahydro-spiro[cyclohexane-1,2'-furo[2,3-*d*][1,3]dioxole]-5'-yl)methyl)piperidin-4-ol (8). Acetic acid (0.05 mL) was added to a solution of 4-(4-chlorophenyl)-4-piperidinol **2** (0.21 g, 1.00 mmol) and (3a'*R*,5'*S*,6'*S*,6a'*R*)-6'-hydroxytetrahydrospiro[cyclohexane-1,2'-furo[2,3-*d*][1,3]dioxole]-5'-carbaldehyde (0.23 g, 0.50 mmol) in MeOH (3 mL). The reaction mixture was stirred overnight, $NaCNBH_3$ (0.18 g, 2.86 mmol) was added and this was stirred for another 2 days. The solvent was evaporated in vacuo, water (10 mL) was added and the water layer was extracted with EtOAc (3 x 5 mL). The combined organic extracts were washed with water (3 x 10 mL) and brine (10 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo. Purification by recrystallization afforded 322 mg (76%) of **8** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.26-1.88 (m, 11H), 1.91-2.18 (m, 2H), 2.42-2.62 (m, 1H), 2.68-2.83 (m, 2H), 2.84-3.00 (m, 1H), 3.05-3.25 (m, 1H), 3.31-3.49 (m, 1H), 4.08-4.21 (m, 1H), 4.28-4.39 (m, 1H), 4.40-4.51 (m, 1H), 5.96 (d, 1H, *J* = 2.8 Hz), 7.21-7.42 (m, 4H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 23.36, 23.68, 24.70, 35.40, 36.16, 37.84, 38.44, 50.43, 51.00, 57.09, 69.29, 76.80, 84.95, 104.15, 111.71, 126.09, 127.95, 132.18, 147.21. Anal. RP-HPLC *Ic*: t_R = 5.69 min (purity 100%), *IVa*: t_R = 12.73 min (purity 100%). HRMS (EI) m/z calcd for $C_{22}H_{30}ClNO_5$: 423.1813; found: 423.1818.

4-(4-chlorophenyl)-1-(((3a*R*,5*R*,6*S*,6a*R*)-6-methoxy-2,2-dimethyltetrahydrofuro[2,3-*d*][1,3]dioxol-5-yl)methyl)piperidin-4-ol fumarate (9). Yield: 189 mg (41%). ^{1}H NMR (DMSO-d_6): δ 1.29 (s, 3H), 1.42 (s, 3H), 1.51-1.69 (m, 2H), 1.82-2.08 (m, 2H), 2.44-2.93 (m, 6H), 3.38 (s, 3H), 3.61-3.74 (m, 1H), 4.19-4.32 (m, 1H), 4.59-4.70 (m, 1H), 5.32 (d, 1H, *J* = 2.4 Hz), 6.61 (s, 2H), 7.31-7.60 (m, 4H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 26.00, 26.49, 36.77, 36.90, 48.81, 49.62, 56.10, 57.47, 69.08, 76.86, 80.59, 84.96, 104.75, 111.27, 126.12, 127.85, 131.99, 134.72, 147.14, 169.01. Anal. RP-HPLC *Id*: t_R = 5.84 min (purity 100%), *IVa*: t_R = 12.04 min (purity 96%). HRMS (EI) m/z calcd for $C_{20}H_{28}ClNO_5$: 397.1656; found: 397.1646.

(3*S*,4*R*,5*R*)-5-((*S*)-2-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-1-hydroxyethyl)-3,4-dihydroxydihydro-furan-2(3*H*)-one (10). Yield: 110 mg (30%). ^{1}H-NMR (MeOH-d_4): δ 1.61-1.80 (m, 2H), 2.05-2.19 (m, 2H), 2.61-3.12 (m, 4H), 3.31-3.50 (m, 2H), 3.71-3.89 (m, 2H), 3.98-4.08 (m, 2H), 7.31 (d, *J* = 8.6 Hz, 2H), 7.49 (d, *J* = 8.6Hz, 2H). ^{13}C NMR (MeOH-d_4): δ 36.91, 49.34, 50.12, 60.29, 71.83, 72.38, 73.11, 126.00, 127.62, 131.79, 146.98, 173.79. Anal. RP-HPLC *If*: t_R = 7.11 min (purity 97%), *IVb*: t_R = 7.94 min (purity 90%). HRMS (ESI) m/z calcd for $C_{17}H_{22}ClNO_6$: 371.1136; found: 371.1136.

4-(4-chlorophenyl)-1-(((2*R*,4*R*,4a*R*,6*S*,8a*S*)-2,6-diphenyltetrahydro-[1,3]dioxino[5,4-*d*][1,3]dioxin-4-yl)methyl)piperidine-4-ol (11). Yield: 342 mg (66%). ^{1}H NMR (DMSO-d_6): δ 1.61-1.90 (m, 2H), 1.91-2.28 (m, 2H), 3.18-4.11 (m, 8H), 4.13-4.37 (m, 1H), 4.40-4.62 (m, 1H), 5.50 (s, 1H), 5.75 (s, 1H), 5.96 (s, 1H), 7.13-7.68 (m, 14H). ^{13}C NMR (DMSO-d_6): δ 34.72, 48.06, 49.36, 55.64, 67.29, 71.88, 74.51, 99.97, 100.79, 126.01, 126.37, 127.88, 127.99, 128.84, 131.32, 136.62, 136.95. Anal. RP-HPLC *Ic* (total run time 25 min): t_R= 13.82 min (purity 100%), *IIIa*: t_R = 13.95 min (purity 100%), *IVa*: t_R = 15.41 min (purity 100%). HRMS (EI) m/z calcd for $C_{30}H_{32}ClNO_5$: 521.1969; found: 521.1956.

General method C for the synthesis of target compounds 12-19. 1-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-7-phenylheptane-1,7-dione (12). EDCI (0.19 g, 1.00 mmol), DIPEA (0.19 g, 1.50 mmol), and HOBT (0.14 g, 1.00 mmol) were added to a solution of 7-oxo-7-phenylheptanoic acid (0.22 g, 1.00 mmol) in DCM (70 mL) and this was stirred for 15 min. 4-(4-Chlorophenyl)-4-piperidinol **2** (0.21 g, 1.00 mmol) was added, and the resulting reaction mixture was stirred for another 18 h. 10% NaOH (2 x 50 mL) was added followed by 0.001 M HCl (2 x 50 mL). The water layer was extracted with DCM (3 x 50 mL), and the combined organic layers were washed with water (2 x 50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. Recrystallization from EtOAc/hexane gave 124 mg (30%) of **12** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.31-1.54 (m, 2H), 1.58-2.09 (m, 8H), 2.38 (t, *J* = 8.6 Hz, 2H), 2.93-3.18 (m, 3H), 3.41-3.65 (m, 1H), 3.66-3.83 (m, 1H), 4.46-4.64 (m, 1H), 7.18-7.60 (m, 7H), 8.93 (d, *J* = 9.4 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 23.77, 25.00, 28.89, 32.92, 37.58, 38.14, 41.70, 70.97, 125.90, 125.98, 127.87, 128.34, 128.43, 132.86, 136.74, 146.24, 171.21, 200.29. Anal. RP-HPLC *Ib*: t_R = 10.20 min (purity 100%), *IVb*: t_R = 10.58 min (purity 100%). HRMS (EI) m/z calcd for $C_{24}H_{28}ClNO_3$: 413.1758; found: 413.1753.

1-(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)-4-(pyren-1-yl)butane-1,4-dione (13). Yield: 180 mg (36%). ^{1}H NMR ($CDCl_3$): δ 1.59-2.11 (m, 5H), 2.79-3.22 (m, 3H), 3.40-3.65 (m, 3H), 3.80-4.02 (m, 1H), 4.48-4.69 (m, 1H), 7.20-7.41 (m, 4H), 7.93-8.30 (m, 2H), 8.52 (d, *J* = 9.0 Hz, 1H), 8.90 (d, *J* = 9.5 Hz, 1H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 27.50, 37.22, 37.49, 37.94, 38.33, 41.57, 70.25, 123.91, 123.97, 124.62, 125.64, 125.92, 126.09, 126.13, 126.90, 127.93, 128.80, 129.07, 130.20, 130.75, 132.12, 132.56, 133.26, 147.16, 169.66, 203.60. Anal. RP-HPLC *IIIa*: t_R= 16.32 min (purity 100%), *IV*: t_R = 14.99 min (purity 100%). HRMS (ESI) m/z calcd for $C_{31}H_{26}ClNO_3$: 495.1601; found:.495.1601.

5,7-Dibromo-3-(4-(4-chlorophenyl)-4-hydroxypiperidine-1-carbonyl)-2*H*-chromen-2-one (14). Yield: 371 mg (68%) ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.57-1.89 (m, 2H), 1.91-2.18 (m, 2H), 3.15-3.46 (m, 2H), 3.53-3.71 (m, 1H), 4.48-4.65 (m, 1H), 7.24-7.41 (m, 4H), 7.58 (d, *J* = 2.2 Hz,

1H), 7.74 (s, 1H), 7.88 (d, *J* = 2.2 Hz, 1H). ^{13}C NMR (DMSO-d_6): δ 37.46, 42.55, 69.70, 110.02, 116.09, 121.18, 126.51, 127.67, 130.28, 130.90, 136.54, 139.94, 147.89, 149.20, 156.58, 161.66. Anal. RP-HPLC *IIa*: t_R= 7.27 min (purity 97%), *IIIa:* t_R= 15.61 min (purity 100%), *IVa*: t_R = 13.50 min (purity 100%). This compound could not be ionized with EI or ESI, so the HRMS value could not be determined.

2-Chloro-5-(4-(4-chlorophenyl)-4-hydroxypiperidine-1-carbonyl)-4-(furan-2-ylmethyl-amino)benzenesulfonamide (15). Yield: 176 mg (34%). ^{1}H NMR ($CDCl_3$): δ 1.39-2.08 (m, 7H), 3.29-3.51 (m, 2H), 3.93-4.40 (m, 3H), 5.07 (s, 2H), 6.11-6.35 (m, 3H), 6.84 (s, 1H), 7.24-7.41 (m, 4H), 7.82 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 37.83, 39.85, 69.88, 107.28, 110.09, 112.64, 116.89, 126.07, 126.82, 127.72, 129.11, 131.89, 133.38, 141.88, 146.99, 148.83, 150.60, 166.96. Anal. RP-HPLC *Ib*: t_R = 4.59 min (purity 97%), *IIb*: t_R = 7.86 min (purity 96%). HRMS (ESI) m/z calcd for $C_{23}H_{23}Cl_2N_3O_5S$: 523.0735; found: 523.0718.

(2-(2-Aminobenzoyl)phenyl)(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)methanone (16). Yield: 276 mg (68%). ^{1}H NMR ($CDCl_3$): δ 1.42-2.25 (m, 5H), 3.02-3.29 (m, 1H), 3.35-3.70 (m, 2H), 4.30-4.53 (m, 1H), 6.48-6.76 (m, 2H), 7.07-7.52 (m, 10H). ^{13}C NMR ($CDCl_3$): δ 37.89, 43.93, 71.47, 115.69, 116.65, 118.07, 125.85, 126.92, 128.23, 128.34, 128.96, 129.81, 132.88, 134.62, 136.36, 139.01, 146.23, 150.78, 169.16, 198.48. Anal. RP-HPLC *Ia*: t_R = 7.63 min (purity 100%), *IIb*: t_R = 10.21 min (purity 99%). HRMS (EI) m/z calcd for $C_{25}H_{23}ClN_2O_3$: 434.1397; found: 434.1385.

(*E*)-4-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-*N*-(4-methoxyphenyl)-4-oxobut-2-enamide (17). Yield: 96 mg (23%). ^{1}H NMR ($CDCl_3$): δ 1.63-1.82 (m, 2H), 2.05-2.27 (m, 2H), 2.64-3.30 (m, 5H), 3.80 (s, 3H), 3.95-4.08 (m, 1H), 6.95 (d, *J* = 9.0 Hz, 2H), 7.15-7.44 (m, 8H). ^{13}C NMR ($CDCl_3$): δ 31.29, 44.37, 55.35, 62.57, 114.36, 123.88, 125.86, 127.51, 128.35, 132.82, 146.32, 159.44, 174.33, 175.36. Anal. RP-HPLC *Ic*: t_R = 7.85 min (purity 100%), *IIb*: t_R = 3.88 min (purity 100%). HRMS (ESI) m/z calcd for $C_{22}H_{23}ClN_2O_4$: 414.1346; found: 414.1358.

(4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl)(1*H*-pyrrol-2-yl)methanone (18). Yield: 212 mg (70%). ^{1}H NMR ($CDCl_3$): δ 1.60-2.19 (m, 8H), 3.31-3.61 (m, 2H), 4.47-4.64 (m, 2H), 6.19-6.27 (m, 1H), 6.51-6.59 (m, 1H), 6.89-6.96 (m, 1H), 7.24-7.42 (m, 4H), 9.48 (br s, 1H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 38.14, 70.51, 108.83, 111.57, 120.67, 124.48, 126.10, 128.00, 132.24, 147.11, 161.50. Anal. RP-HPLC *Ib*: t_R = 4.66 min (purity 98%), *IIb*: t_R = 4.06 min (purity 100%). HRMS (EI) m/z calcd for $C_{16}H_{17}ClN_2O_2$: 304.0979; found: 304.0992.

6-(4-(4-Chlorophenyl)-4-hydroxypiperidine-1-carbonyl)-2*H*-thiazolo[3,2-*a*]pyrimidin-5(3*H*)-one (19). Yield: 105 mg (27%). ^{1}H NMR ($CDCl_3$): δ 1.58-1.82 (m, 2H), 1.85-2.11 (m, 2H), 3.16-3.33 (m, 1H), 3.41-3.63 (m, 4H), 4.43-4.72 (m, 3H), 7.3-7.48 (m, 4H), 7.94 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 23.94, 35.30, 35.75, 36.26, 41.15, 46.58, 68.86, 117.02, 123.35, 123.56, 126.00, 130.54,

143.72, 151.73, 155.20, 160.87, 164.50. Anal. RP-HPLC *Id*: t_R = 9.84 min (purity 100%), *IIIa*: t_R = 12.42 min (purity 100%), *IVa*: t_R = 10.78 min (purity 100%). HRMS (EI) m/z calcd for $C_{18}H_{18}ClN_3O_3S$: 391.0757; found: 391.0744.

General method D for the synthesis of target compound 20. 4-(4-Chlorophenyl)-1-(4-(morpholinosulfonyl)phenylsulfonyl)-piperidin-4-ol (20). A solution of 4-(4-chlorophenyl)-4-piperidinol **2** (0.21 g, 1.00 mmol) and Et_3N (0.20 g, 2.00 mmol) in DCM (10 mL) was added to a solution of 4-(morpholinosulfonyl)benzene-1-sulfonyl chloride (0.33 g, 1.01 mmol) in DCM (10 mL). The reaction mixture was stirred at room temperature for 3 h, quenched with water (20 mL) and the water layer was extracted with DCM (3 x 50 mL). The combined organic layers were washed with water (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure. Recrystallization from MeOH/hexane gave 105 mg (68%) of **20** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.72-1.85 (m, 2H), 2.08-2.29 (m, 2H), 2.79-2.94 (m, 2H), 3.03-3.15 (m, 4H), 3.69-3.83 (m, 6H), 7.25-7.41 (m, 4H), 7.90-8.05 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 34.66, 41.99, 45.63, 65.04, 68.33, 126.49, 127.65, 128.45, 130.99, 138.41, 139.63, 147.55. Anal. RP-HPLC *Ib*: t_R = 5.59 min (purity 100%), *IIb*: t_R = 14.31 min (purity 100%). HRMS (ESI) m/z calcd for $C_{21}H_{25}ClN_2O_6S_2$: 500.0843; found: 500.0865.

Pharmacology

[^{125}I]CCL5 binding experiments and [^{3}H]inositol phosphate accumulation assays were performed as previously described in the literature. [6]

References

1. Gurrath, M. Peptide-binding G protein-coupled receptors: new opportunities for drug design. *Curr. Med. Chem.* **2001**, *8*, 1605-1648.
2. Onuffer, J. J.; Horuk, R. Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol. Sci.* **2002**, *23*, 459-467.
3. Vischer, H. F.; Vink, C.; Smit, M.J. A viral conspiracy: hijacking the chemokine system through virally encoded pirated chemokine receptors. *Curr. Top. Microbiol. Immunol.* **2006**, *303*, 121-154.
4. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
5. Hulshof, J. W.; de Esch, I. J. P.; Leurs, R. Synthesis and structure-activity relationship of the first nonpeptidergic inverse agonists for the human cytomegalovirus encoded chemokine receptor US28. *J. Med. Chem.* **2005**, *48*, 6461-6471.
6. Hulshof J. W.; Vischer, H. F.; Verheij, M. H.; Fratantoni, S. A.; Smit, M. J.; de Esch, I. J. P.; Leurs, R. Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28. *Bioorg. Med. Chem.* **2006**, *14*, 7213-7230.
7. Rose, S. Statistical design and application to combinatorial chemistry. *Drug Discov. Today* **2002**, 7, 133–138.
8. Schneider, G.; Böhm, H.-J. Virtual screening and fast automated docking methods. *Drug Discov. Today* **2002**, *7*, 64–70.
9. Orry, A. J.; Abagyan, R. A.; Cavasotto, C. N. Structure-based development of target-specific compound libraries. *Drug Discov. Today* **2006**, 11, 261-266.

10. Miller, J. L. Recent developments in focused library design: targeting gene-families. *Curr. Top. Med. Chem.* **2006**, *6*, 19-29.
11. Gribbon, P.; Sewing, A. High-throughput drug discovery: what can we expect from HTS? *Drug Discov. Today* **2005**, *10*, 17-22.
12. Venkatesh, S.; Lipper, R. A. Role of the development scientist in compound lead selection and optimization. *J. Pharm. Sci.* **2000**, *89*, 145-154.
13. Rishton, G. M. Nonleadlikeness and leadlikeness in biochemical screening, *Drug Discov. Today* **2003**, *8*, 86-96.
14. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3-25.
15. Martin, E. J.; Critchlow, R. E. Beyond mere diversity: tailoring combinatorial libraries for drug discovery. *J. Comb. Chem.* **1999**, *1*, 32-45.
16. Roche, O.; Schneider, P.;Zuegge, J.; Guba, W.; Kansy, W.; Alanine, A.; Bleicher, K.; Danel, F.; Gutknecht, E.-M.; Rogers-Evans, M.; Neidhart, W.; Stalder, H.; Dillon, M.; Sjögren, E.; Fotouhi, N.; Gillespie, P.; Goodnow, R.; Harris, W.; Jones, P.; Taniguchi, M.; Tsujii, s.; von der Saal, w.; Zimmermann, G.; Schneider, G. Development of a virtual screening method for identification of "frequent hitters" in compound libraries. *J. Med. Chem.* **2002**, *45*, 137-142.
17. Oprea, T. I. Current trends in lead discovery: are we looking for the appropriate properties? *J. Comput. Aided. Mol. Des.* **2002**, 16, 325-334.
18. Ajay, A.; Walters, W. P.; Murcko, M. A. Can we learn to distinguish between "drug-like" and "nondrug-like" molecules? *J. Med. Chem.* **1998**, *41*, 3314-3324.
19. Sadowski, J.; Kubinyi, H. A scoring scheme for discriminating between drugs and nondrugs. *J. Med. Chem.* **1998**, *41*, 3325-3329.
20. Frimurer, T. M.; Bywater, R.; Naerum, L.; Lauritsen, L. N.; Brunak, S. Improving the odds in discriminating "drug-like" from "non drug-like" compounds. *J. Chem. Inf. Comput. Sci.* **2000,** *40*, 1315-1324.
21. Hann, M.; Hudson, B.; Lewell, X.; Lifely, R.; Miller, L.; Ramsden, N. Strategic pooling of compounds for high-throughput screening. *J. Chem. Inf. Comput. Sci.* **1999**, *39*, 897-902.
22. Baurin, N.; Baker, R.; Richardson, C.; Chen, I.; Foloppe, N.; Potter,A.; Jordan, A.; Roughley, S.; Parratt, M.; Greaney, P.; Morley, D.; Hubbard, R. E. Drug-like annotation and duplicate analysis of a 23-supplier chemical database totalling 2.7 million compounds. *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 643-651.
23. Yasri, A.; Berthelot, D.; Gijsen, H.; Thielemans, T.; Marichal, P.; Engels, M.; Hoflack, J. REALISIS: a medicinal chemistry-oriented reagent selection, library design, and profiling platform, *J. Chem. Inf. Comput. Sci.* **2004**, *44*, 2199-2206.
24. Thurkauf, A.;Yuan, J.; Chen, X.; Wasley, J. W. F.; Meade, R.; Harris Woodruff, K.; Huston, K.; Ross, P. C. l-Phenyl-3-(aminomethyl)pyrroles as potential antipsychotic agents. Synthesis and dopamine receptor binding. *J. Med. Chem.* **1995**,*38*, 4950-4952.
25. Mattson, R. J.; Pham, K. M.; Leuck, D. J.; Cowens, K. A. An improved method for reductive alkylation of amines using titanium(IV) isopropoxide and sodium cyanoborohydride. *J. Org. Chem*. **1990**, *55*, 2552-2554.
26. Palani, A.; Shapiro, S.; Josien, H.; Bara, T.;Clader, J. W.; Greenlee, W. J.; Cox, K.; Strizki, J. M.; Baroudy, B. M. Synthesis, SAR, and biological evaluation of oximino-piperidino-piperidine amides. 1. Orally bioavailable CCR5 receptor antagonists with potent anti-HIV activity. *J. Med. Chem.* **2002**, *45*, 3143-3160.
27. Fecik, R. A.; Frank, K. E.; Gentry, E. J.; Menon, S. R.; Mitscher, L. A.; Telikepalli, H. The search for orally active medications through combinatorial chemistry. *Med. Res. Rev.* **1998**, *18*, 149-185.
28. Bhattacharyya, S. Reductive alkylations of dimethylamine using titanium(IV) isopropoxide and sodium borohydride: an efficient, safe, and convenient method for the synthesis of N,N-dimethylated tertiary amines. *J. Org. Chem.* **1995**, *60*, 4928-4929.
29. Chandrasekhar, S.; Reddy, C. R.; Moinuddin, A. A single step reductive amination of carbonyl compounds with polymethylhydrosiloxane-Ti(OiPr)$_4$. *Syn. Lett.* **2000**, *11*, 1655-1657.

Chapter 5

The quest for novel ligands for the constitutively active receptor US28

Janneke W. Hulshof, Henry F. Vischer , Silvina A. Fratantoni, Martine J. Smit, Rob Leurs and Iwan J. P. de Esch

Abstract

In our quest to identify novel ligands for the viral-encoded receptor US28, we performed an extensive screening. Compounds from our in-house compounds database were selected based on their molecular similarity to structures of known ligands acting on US28. The screening of the selected compounds resulted in the identification of several inverse agonists, which can be considered as new starting points for hit optimization programs. Furthermore, we disclose 5-(3-(4-methylpiperazin-1-yl)propyl)-5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine (**35**) and 2-phenyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizine (**44**) as the very first nonpeptidergic neutral antagonists acting on a viral-encoded receptor. The discovery of novel ligands with unique pharmacological profiles gives us unprecedented opportunities to further investigate the role of constitutive signaling of US28 during viral infection.

Introduction

Human cytomegalovirus (HCMV) is a DNA virus that encodes homologues of host G protein-coupled receptors (GPCRs) for viral replication and persistence. HCMV is a widespread pathogen and infection with the virus is associated with different pathologies, such as the development of vascular diseases, cancer, chronic inflammation and birth defects.[1] Moreover, co-infection of the virus with human immunodeficiency virus (HIV) has been shown to cause an increased risk of disease progression to acquired immunodeficiency syndrome (AIDS) and dementia in HIV patients.[2]

At present, US28 is the most extensively characterized receptor encoded by HCMV and many important roles during viral infection have been attributed to this GPCR. The receptor shows sequence homology with the CCR1 chemokine receptor family[3] and can bind several CC chemokines, such as CCL2, CCL3, CCL4 and CCL5 with high affinity,[3-5] suggesting that US28 acts as a chemokine scavenger by sequestering these chemokines from the extracellular environment via endocytosis. This mode of action putatively represents a mechanism to escape immune surveillance after HCVM infection.[6-8]

Unlike the human chemokine receptors, US28 signals in a constitutively active manner through different signaling pathways in both transiently transfected COS-7 cells as well as in HCMV-infected cells.[9,10] Although constitutive signaling seems to be a general characteristic of viral-encoded GPCRs, the biological relevance has not been elucidated yet. However, the importance of this ligand-independent signaling has been demonstrated for the HHV-8 or Kaposi's sarcoma-associated herpesvirus (KSHV) encoded GPCR ORF74. This receptor is a viral oncogene that activates multiple intracellular signaling pathways in a constitutively active manner resulting in the production and secretion of VEGF (vascular endothelial growth factor) and thereby causing cellular transformations that can develop into highly vascularized Kaposi's sarcoma-like lesions in transgenic mice.[11,12] Interestingly, it has recently been shown that US28 acts as a viral oncogene as well, because expression of US28 in NIH-3T3 cells induces a transformed phenotype and promotes tumorigenesis in mice.[13] The constitutive activity of US28 seems to be of importance in the early onset of tumorigenesis

as can be deduced from delayed and attenuated tumor formation by the US28-R129A mutant receptor, which lacks constitutive signaling. Constitutive activity can be inhibited by ligands that behave as so-called inverse agonists. The discovery of such molecules for US28 will help to unravel the role of US28 in viral pathology and cancer.

The identification of small molecules as modulators of protein activity and the subsequent hit optimization are key activities in modern drug discovery. During the last decade, many hits with the potential to progress to viable drug candidates have emerged from high throughput screening (HTS) of corporate compound collections.[14,15] However, this approach is not always succesful. In a HTS campaign hundreds of thousands of compounds are tested, but this strategy is expensive and does frequently not lead to new hits that have the potential to become optimized. Therefore, other strategies have emerged to discover leads, such as the development of focused libraries that are generated on the basis of structural information about the ligands and/or the biological target or the generation of novel ideas from small molecules reported in the literature.[16,17]

In our quest to identify novel ligands for the viral-encoded receptor US28 we have undertaken screening efforts. For this, we have used our own proprietary compound collection. We based our selection of compounds on the basis of molecular structure of known US28 ligands (Figure 1). So far, only few US28 ligands have been identified. Recently, VUF2274 (**1**) was identified as the first inverse agonist acting on US28[10] and the synthesis and pharmacological characterization of an extensive series of analogues revealed the very first structure-activity relationships for inverse agonism on US28.[18,19] Replacement of the 4-hydroxy group of compound **1** into the methylamine group in VUF6966 (**2**) resulted in a 6-fold increase in binding affinity and this compound is, to our knowledge, one of the highest affinity inverse agonist acting on US28 currently known.[19]

Moreover, several ligands have been reported by Chemocentryx Inc. in the patent literature. These molecules were claimed to block dissemination of CMV in a host by reversibly inhibiting chemokine binding to US28 in the micromolair range, such as a series of piperazinyldibenzothiepins, represented by the serotonin receptor antagonists methiothepin (**3**) and octoclothepin (**4**) and a large series of bicyclic structures, exemplified by VUF6045 (**5**).[20,21] Furthermore, a series of benzamides was mentioned to interact with US28, and of this series the commercially available dopamine receptor ligand S-(-)-IBZM (**6**) was the only compound of which results were shown.[21] Interestingly, all these molecules induce a rise in intracellular Ca^{2+}, thereby acting as agonists on US28.

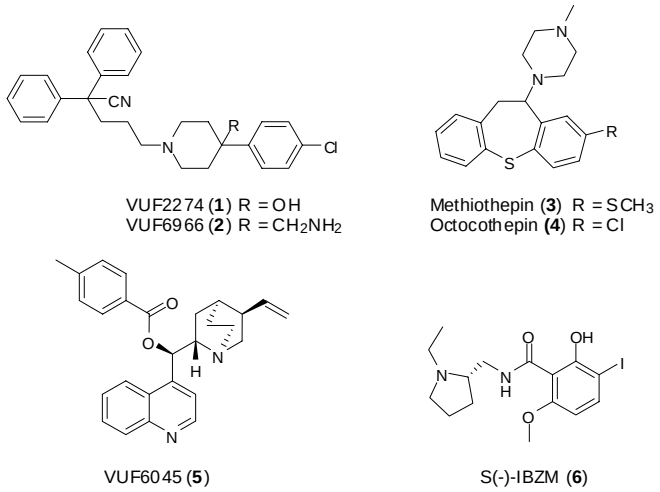


Figure 1. Chemical structures of the known US28 ligands **1**-**6**.

In this study, we will disclose the results from our ligand-based screening efforts. These novel ligands will give us more insights in the structural requirements that are important for binding to US28.

Results and discussion

The compounds were selected by searching the in-house database for compounds bearing structural similarity, i.e. substructure search, of the known US28-ligands **1**-**6**. Ligand substructure similarity searches were performed using the database management system (ChemFinder Std. 7.0, CambridgeSoft

Corporation). Next to the substructure searches, several database compounds were selected for screening based on a subjective structural similarity assessment after visual inspection.

More than two hundred compounds were screened at a single concentration of 10 μM for their efficacy by measuring their influence on the [^{3}H]inositol phosphate production, whereas the binding affinity was determined by their potential to displace [^{125}I]CCL5 binding to US28. A schematic representation of the single point measurements is shown in Figure 2.

A. Results of all compounds that show structural similarity to compounds **3** and **4**.

B. Results of the compounds selected by a subjective structural similarity assessment after visual inspection.

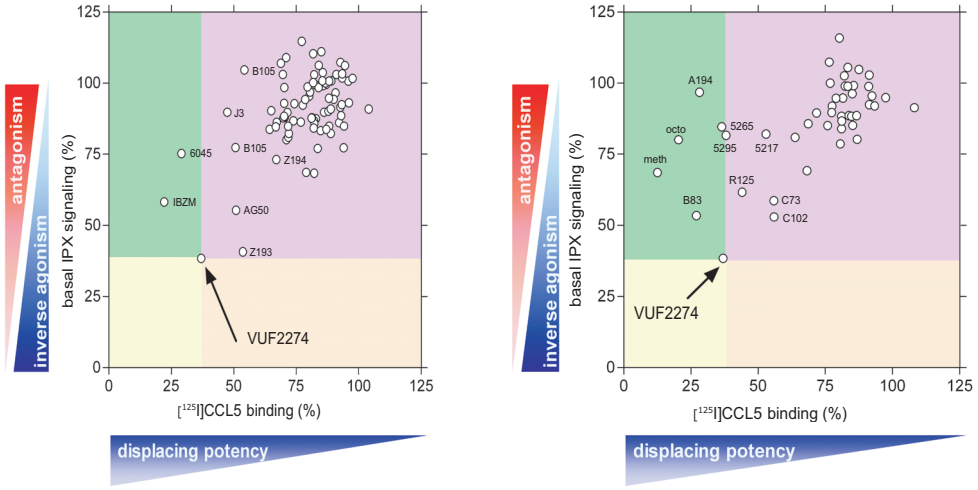


Figure 2. Schematic representation of all single points measurements. Data are represented as percentage of the [^{125}I]CCL5 binding (x-axis) against the percentage of the basal [^{3}H]inositol phosphate (IPX) signaling (y-axis). The average of at least three independent experiments is shown. Classification of the compounds: green shows the compounds that displace more [^{125}I]CCL5 from the receptor than **1**, but their influence on the basal IPx signaling (inverse agonistic properties) is lower. In the purple part the compounds are shown that displace less [^{125}I]CCL5 from the receptor than **1** and that have lower inverse agonistic properties. No compounds were found in the yellow or pink part, in which the compounds with better inverse agonistic properties than compound **1** would have been found.

As can be seen in Figure 2, several molecules were identified as novel ligands for US28. For all compounds that showed more than 50% displacement of [^{125}I]CCL5 the IC_{50} and EC_{50} values were obtained. Moreover, the in-house database was searched for structural analogues of these hits. The results of this comprehensive study are summarized in Table 1-5.

Firstly, the results of methiothepin (**3**) and octoclothepin (**4**) and their analogues are shown in Table 1. It is noted that the IC_{50} value of compound **3** that has been previously generated in literature[20] by performing radiodisplacement studies using a different labeled chemokine, namely [^{125}I]CX3CL1, was comparable to the IC_{50} value generated in our studies with [^{125}I]CCL5. Interestingly, compound **3** shows a 2-fold higher binding affinity than compound **2** and even a 10-fold higher binding affinity than compound **1**.[19] Replacement of the thiomethylene group of **3** by a chloro atom in compound **4** resulted in an affinity comparable to that of compound **1**.

In the patent literature compounds **3** and **4** have been demonstrated to act as agonists in cytoplasmic calcium mobilization experiments in US28-expressing HEK293 cells by inducing a rise in intracellular Ca^{2+}. Surprisingly, in SVEC4-10 cells these compounds act as inverse agonists by inhibiting the US28-mediated constitutive activation of PLC in a dose-dependently manner. To investigate if this discrepancy in the different functional assays was dependent on the cell line, the influence of methiothepin on the Ca^{2+} levels in US28-expressing SVEC4-10 cells was determined as well (See Figure 3).

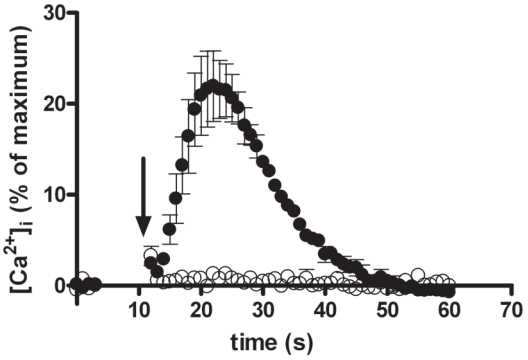
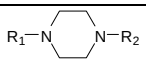


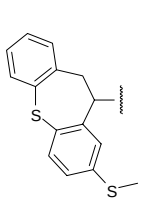
Figure 3. Methiothepin-induced [Ca^{2+}] mobilization in SVEC4-10 cells. Stably US28-expressing (black dots) and mock transfected (open dots) cells were loaded with Fluo 4-AM and exposed to 10 μM methiothepin. The black arrow indicates the time at which methiothepin (10 μM) was added to the cells. Data are normalized to maximum fluorescence signal resulting from addition of Triton X-100 (100%). A representative curve of three independent experiments is shown.

The applicability of this assay is very limited, because the presence of DMSO hampers the Ca^{2+} measurements and only methiothepin can be dissolved in water. Thus, the influence on the Ca^{2+} efflux cannot be determined for any other compound. Intriguingly, in the SVEC4-10 cells methiothepin also shows an increase in Ca^{2+} levels. Therefore, we conclude that methiothepin is a protean ligand on US28. Protean ligands are compounds that have multiple functional properties on the same receptor measured from the same cells, e.g. inverse agonism in one particular pharmacological assay and neutral antagonism in another one.[23-26] Protean ligands have also been identified for, e.g. the histamine H_3 receptor,[27,28] the α_{2A}-adrenergic receptor[29,30] and the human CB_2 cannabinoid receptor.[31] Although protean ligands have been characterized pharmacologically, the molecular basis of the mode of action on molecular level has not been elucidated yet.[24,32] Additionally, the therapeutic potential of protean agonists is not clear yet. In theory, it would be possible to obtain tissue selectivity of drugs by regulation of receptor function with protean ligands that have agonistic properties in one tissue, while being inverse agonists or neutral antagonists in other tissue.[25]

Although the single point measurements of compounds **7** and **10**-**17** provide us with limited pharmacological data, we like to describe some preliminary structure-activity relationship (SAR) studies. Clozapine (**7**) is a dopamine antagonist that is widely used as an atypical antipsychotic drug with the advantage that it induces a low incidence of undesirable motor side effects. This compound exhibits an approximately 10-fold selectivity for D_4 versus D_2 dopamine receptors, but it also has a high affinity for a variety of other receptors, such as serotonergic receptors (human 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors), adrenergic receptors (human α_{2B} and α_{2C}), the histamine H_1 and H_4 receptor and the muscarinic M_1 receptor.[33-36] Interestingly, compound **7** is also able to displace [^{125}I]CCL5 binding from US28 at a concentration of 10 μM. Elongation of the R_2 group into an ethyl- (**8**) or butyl moiety (**9**) results in compounds with a binding affinity in the low micromolair range. Nevertheless, the inverse agonistic properties of compounds **7**-**9** on US28 are negligible. Interestingly, changing the chloro substituent from the 8- to the 2-position (compound **10**) is detrimental for the binding affinity.

Table 1. Chemical structures and pharmacological properties of compounds **3**-**4** and **7**-**17** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.





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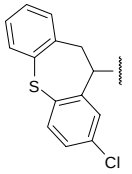

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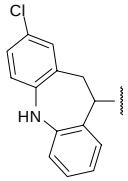

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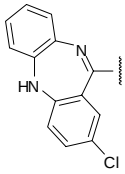

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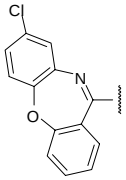

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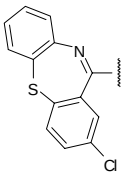

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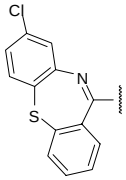

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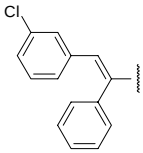


H

no.	Name	R_1	R_2	IC_{50} (μM)[a]	EC_{50} (μM)[b]
3	Methiothepin	A	$-CH_3$	0.41 (0.37 – 0.45)	7.6 (4.5 – 10.7)
4	Octoclothepin	B	$-CH_3$	4.9 (1.6 – 8.1)	12.1 (5.6 – 18.6)
7	Clozapine	C	$-CH_3$	47% [c]	11% [c]
8	VUF5265	C	$-CH_2CH_3$	3.8 (2.6 – 5.1)	16% [c]
9	VUF5295	C	$-(CH_2)_3CH_3$	4.7 (3.2 – 6.2)	19% [c]
10	Loxapine	D	$-CH_3$	13% [c]	0% [c]
11	VUF6879	E	$-CH_3$	19% [c]	22% [c]
12	VUF6876	E	$-CH_2CH_3$	38% [c]	0% [c]
13	Entumine	F	$-CH_3$	28% [c]	0% [c]
14	HUF 2118	G	$-CH_3$	47% [c]	0% [c]
15	VUF5216	H	$-CH_3$	13%[c]	20% [c]
16	VUF5217	H	$-(CH_2)_4Ph$	47% [c]	18% [c]
17	VUF5218	H	$-(CH_2)_4OH$	13% c	0%[c]

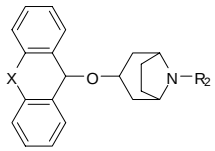
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Results of the single point measurements at a concentration of 10 μM.

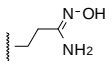
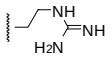
Recently, VUF6879 (**11**) and VUF6876 (**12**) have been published in our group as histamine H_4 receptor ligands.[36] On US28, both compounds are almost

completely inactive. Entumine or clothiapine (**13**) is a drug used for the treatment of psychotic disorders, anxiety, drug dependence and alcoholism. Like clozapine, this compound has a promiscuous binding profile by interacting with many receptors.[37] However, the compound does not show any binding affinity on US28. Moving the chloro substituent from the 2- to the 8-position as in compound **14** results in a slight increase in binding affinity. Compounds **15**-**17** have a high flexibility due to their open structure. As can be seen in Table 1, there is a slight preference for a ω-phenylbutyl group at the R_2 position (compare compound **16** with **15** and **17**), but in the functional assay all three compounds show no or only a small inhibition of the constitutive PLC signaling.

As outlined in Table 2, the database screening reveals the two structurally related analogues B83 (**18**) and C102 (**19**) as new inverse agonists for US28.

Table 2. Chemical structures and pharmacological properties of compounds **18**-**27** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



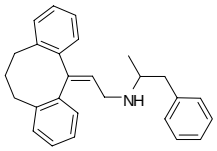
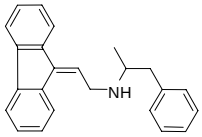
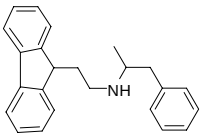
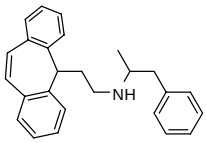
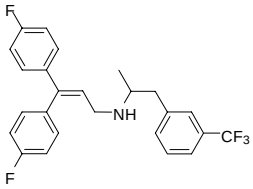
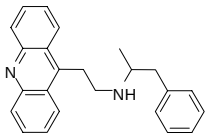
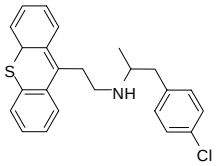
no.	Name	X	R_1	IC_{50} (μM)[a]	EC_{50} (μM)[b]
18	B83	CH_2-CH_2	$(CH_2)_7CH_3$	5.6 (3.4 – 7.8)	4.9 (3.7 – 6.2)
19	C102	CH_2-CH_2	$(CH_2)_2Ph$	11.9 (8.7 – 15.1)	4.3 (3.5 – 5.1)
20	C92	CH_2-CH_2	CH_2Ph	10.5 (8.3 – 12.6)	8.8 (5.9-11.7)
21	A138	CH_2-CH_2	CH_3	15%[c]	0%[c]
22	B143	CH_2-CH_2	CH_2CH_3	7%[c]	0%[c]
23	B93	CH_2-CH_2		0%[c]	0%[c]
24	B98	CH_2-CH_2		34%[c]	0%[c]
25	U238	CH=CH	$CH(CH_3)_2$	13%[c]	3%[c]
26	B150	CH=CH	$(CH_2)_2CH_3$	18%[c]	0%[c]
27	U261	CH=CH	cyclohexyl	8.3 (6.8 – 9.8)	5.8 (3.3 – 8.3)

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Result of the single point measurements at a concentration of 10 μM.

These compounds and some of their analogues were previously synthesized to investigate their potential in chronic non-specific respiratory disorders as well as allergic conditions.[38] Of the analogues mentioned in Table 2, only compound **22** showed promising in vitro activities in this study. The pharmacological results of compounds **18**-**27** on US28 are depicted in Table 2. Replacement of the octyl group of **18** by the phenetyl group in **19** causes the binding affinity to drop slightly, and the inverse agonistic properties are comparable. Removal of a CH_2 group between the bridged piperidine moiety and the phenyl ring as in compound **20** does not change the affinity on US28. In contrast, small methyl and ethyl chains, as in **21** and **22**, are not allowed to maintain affinity or efficacy. Both the *N*-hydroxypropionimidamide group in **23** as the ethyl guanidine moiety in **24** are not allowed for US28 activity. Compounds **25**-**27** differ from the other analogues, due to the presence of a double bond in the tricyclic structure. As demonstrated for compounds **21** and **22**, the small alkyl chains in **25** and **26** are detrimental for both affinity and activity. However, the cyclohexyl group in compound **27** is allowed and results in a binding affinity and efficacy in the same order as compounds **18**-**20**.

The structures and pharmacological properties of compounds R125 (**28**), C73 (**29**) and their analogues are depicted in Table 3. These compounds have comparable binding affinities and efficacies although their tricyclic moieties are quite different in respect to the rigidity and the conformation of the two aromatic rings. The tricyclic moiety of compound **28** is bended due to the presence of three carbon atoms between the two phenyl rings, while the tricyclic ring system of **29** is nearly planar, as determined via a conformational analysis study using the molecular modelling program MOE (molecular operating environment version 2004.03, results not shown). Additionally, saturation of the double bond of **29** as in compound **30** results in a compound with a comparable affinity, and also the introduction of an unsaturated bridge between the two phenyl rings in **31** does not improve the affinity or efficacy. Compounds **32** and **33** both have a *m*-trifluoromethyl group in the phenyl ring of the phenethyl group attached to the nitrogen atom.

Table 3. Chemical structures and pharmacological properties of compounds **28**-**34** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.

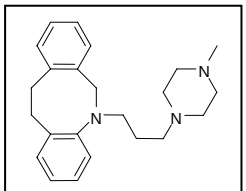
no.	Name	Structure	IC_{50} (μM)[a]	EC_{50} (μM)[b]
28	R125		4.2 (3.6 – 4.8)	39%[c]
29	C73		7.0 (5.9 – 8.1)	42% [c]
30	A185		5.2 (4.4 – 6.0)	10.9 (6.0 – 15.8)
31	C162		3.7 (3.2 – 4.2)	5.2 (3.2 – 7.1)
32	Q113		39% [c]	38% [c]
33	F10a		5.5 (4.5 – 6.5)	34% [c]
34	K15		2.6 (2.0 – 3.2)	21% [c]

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Results of the single point measurements at a concentration of 10 μM.

As can be seen in Table 3, there is a slight preference for the tricyclic system of **33** compared to the 4,4'-difluorophenyl group in **32**. A 2-fold increase in binding affinity compared to compound **33** is observed for compound **34**, in which the

m-trifluoromethyl is replaced by a *p*-chloro substituent and the two phenyl rings of the tricyclic system are connected by a sulphur bridge.

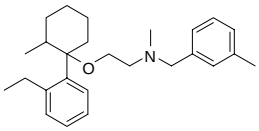
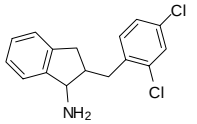
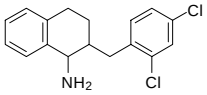
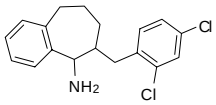
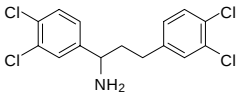
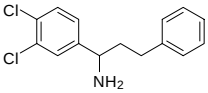
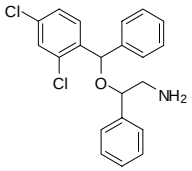
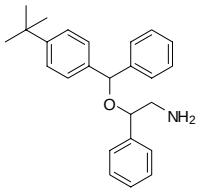
A194 (**35**) resulted from of the database screening as a potential new lead compound on US28. The synthesis of this tricyclic imipramine analogue has been previously described in the literature, but no pharmacological data of this compound are known.[39,40] In this study, compound **35** is able to displace [^{125}I]CCL5 binding from US28 in a dose-dependent manner with an IC_{50} value of 4.9 μM. However, it does not influence the basal signaling of the receptor, thereby acting as a neutral antagonist. To our knowledge, this compound is the very first small nonpeptidergic neutral antagonist acting on a viral-encoded receptor. The SAR of **35** and its analogues will be discussed elsewhere (see Chapter 6).



Compound **35**.

As mentioned earlier, compounds **5** and **6** originated from the patent literature.[21,22] Unfortunately, the in-house database did not contain any analogues of these compounds. However, after visual inspection of the database several compounds were selected for screening subjectively inspired by the substructure of compound **6**, such as J3 (**36**) and Z193 (**37**). Structurally related compounds of **36** and **37** were found as well and the pharmacological evaluation of these compounds resulted in interesting preliminary structure-activity relationships (Table 4). Enlargement of the cyclopentyl group of **37** with one carbon atom as in compound **38** does not influence the affinity or efficacy on US28. Additionally, the introduction of an additional CH_2 group as in **39** results in a binding affinity comparable to that of compound **37** and **38**, but the efficacy of this compound is much lower with only a 26% inhibition of the inositol phosphate production at a concentration of 10 μM. In compound **40** the amine group is not attached to a rigid ring structure as in compounds **37**-**39**, but to the flexible propyl chain resulting in more conformational freedom. This results in an activity comparable to that of compounds **37** and **38**. Interestingly, removal of the chloro atoms from one of the phenyl rings in compound **41** is detrimental for US28 affinity. This suggests that 3,4-dichloro substitution in that parrticular phenyl ring is of importance.

Table 4. Chemical structures and pharmacological properties of compounds **6** and **36-43** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.

no.	Name	Structure	IC_{50} (μM)[a]	EC_{50} (μM)[b]
36	J3		12.9 (5.4 – 20.4)	10% c
37	Z193		9.5 (6.8 – 12.3)	11.1 (8.3 – 13.8)
38	Z172		8.5 (6.8 – 10.2)	12.6 (10.0 – 15.1)
39	Z194		11.8 (11.5 – 12.0)	26% [c]
40	Z150		7.5 (5.0 -10.0)	11.7 (9.3 – 14.1)
41	Z9		5% [c]	34% [c]
42	Z130		9.2 (6.3 – 12.0)	10.2 (7.2 – 9.5)
43	Z131		8.2 (6.3 – 10.0)	8.4 (7.2 – 9.5)

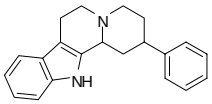
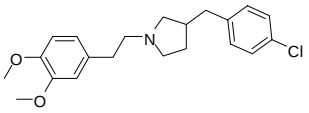
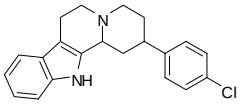
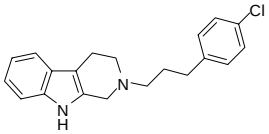
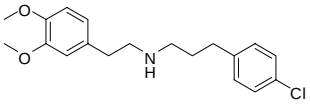
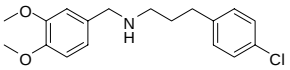
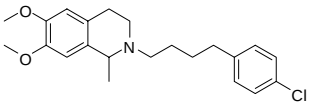
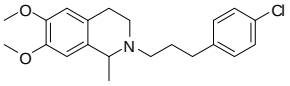
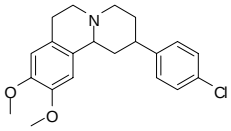
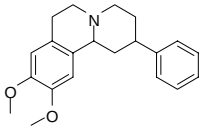
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Results of the single point measurements at a concentration of 10 μM.

Compound **42** has a binding affinity and an efficacy in the same range as compounds **37** and **38** and replacement of the two chloro atoms in the phenyl ring by a *t*-butyl group at the 4-position in **43** does not result in a compound with a higher affinity.

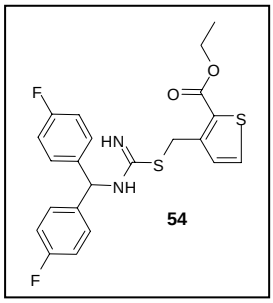
Another new group of compounds that resulted from the database screening were B191 (**44**) and B105 (**45**) and their analogues (Table 5). Of this series, compounds **45** and **48**-**53** were previously synthesized to investigate their potential analgesic activity. Although some of these compounds showed significant analgesic and sedative effects, none of them was selected for further investigation.[41]

As can be seen in Table 5, most compounds of these series show weak if any activity on US28. However, the pharmacological data of compound **44** on US28 shows that this molecule acted as a neutral antagonist by displacing [^{125}I]CCL5 binding from US28 with an IC_{50} value of 10.4 μM without influencing the basal signaling of the receptor. Surprisingly, introduction of a chloro substituent at the *para* position of the phenyl ring attached to the cyclic moiety in compound **46** has a drastic effect on the affinity of the compound. Increasing the flexibility as in compound **47** is detrimental for the binding affinity as well. Compound **45** has a binding affinity comparable to that of compound **44** and shows a low inhibition of the inositol phosphate production. Unfortunately, all tested analogues show a low if any activity on US28. Thus, increasing the flexibility as in compounds **48** and **49**, which has one carbon atom less between the phenyl ring and the amine group compared to **48**, is detrimental for affinity. Additionally, incorporation of the nitrogen atom in a 1-methyl-tetrahydroisoquinoline moiety (**50**) is not allowed to maintain affinity. In compounds **51** and **52** the nitrogen atom is incorporated in a tetrahydroisoquinoline moiety, but in **51** the 4-chlorophenyl group is linked to the amine group by a propyl chain, while in **52** the linker is part of a tetrahydroisoquinoline containing tricyclic moiety. Both these compounds do not show a significant affinity or efficacy on US28. Removal of the chloro substituent in the aromatic ring of compound **52**, resulting in compound **53**, does not improve the activity of the compound either.

Table 5. Chemical structures and pharmacological properties of compounds **44**-**53** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.

no.	Name	Structure	IC_{50} (μM)[a]	EC_{50} (μM)[b]
44	B191		10.4 (6.8 – 14.1)	0% [c]
45	B105		8.8 (3.5 – 14.1)	23%[c]
46	C86		26%[c]	35% [c]
47	B126		3% [c]	18% [c]
48	B73		7% [c]	12% [c]
49	B157		0% [c]	4% [c]
50	B115		30% [c]	12% [c]
51	B81		4% [c]	0% [c]
52	B166		34% [c]	32% [c]
53	A40		31% [c]	0% [c]

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production. [c] Results of the single point measurements at a concentration of 10 μM.



54

AG50 (**54**) was identified as a new inverse agonist acting on US28. Compound **54** is a promising ligand with IC_{50} and EC_{50} values of 12.6 μM (11.5 – 13.8) and 4.7 μM (4.1 - 5.4 μM). Unfortunately, no analogues of **54** were present in the database and therefore no preliminary indication of SAR can be given.

Conclusions

In summary, we have described our search for new ligands acting on the constitutively active viral-encoded receptor US28. This was achieved by a ligand-based screening with a selection of compounds from our in-house database. The database screen revealed several interesting inverse agonists, which can all serve as new starting points for lead optimization programs. Several structural diverse novel inverse agonists were discovered. In addition, two compounds with a different pharmacological profile were identified, namely compounds **35** and **44**. Both compounds were able to bind to the receptor with IC_{50} values in the micromolar range, but they did not influence the basal signaling of the receptor, thereby acting as neutral antagonists on US28. Thus, we identified the very first neutral antagonists on the viral-encoded receptor US28. Interestingly, the availability of inverse agonists, that are able to inhibit the constitutive signaling of the receptor, and neutral antagonists, that do not influence the basal signaling, gives us the possibility to further investigate the significance of constitutive US28-signaling and the role of the receptor during HCMV infection.

Experimental

Compounds **3**, **4**, **6**, **7** and **10** were purchased from Sigma-Aldrich Co. (USA). Compounds **11** and **12** were recently reported in the literature.[36] Compounds **13** and **14** were kindly donated by Dr. Aebischer from the Sandoz research institute in Bern. The purity of all compounds from the in-house database was ≥95%, as determined via LC-MS measurements. Transient and stable expression of US28, the [^{125}I]CCL5 binding experiments and the [^{3}H]inositol phosphate accumulation assay were performed as previously described.[19]

References

1. Landolfo, S.; Gariglio, M.; Gribaudo, G.; Lembo, D. The human cytomegalovirus. *Pharmacol. Ther.* **2003**, *98*, 269–297.
2. Deayton, J. R.; Sabin, C. A.; Johnson, M. A.; Emery, V. C.; Wilson, P.; Griffiths, P. D. Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. *Lancet* **2004**, *363*, 2116–2121.
3. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
4. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
5. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.
6. Bodaghi, B.; Jones, T. R.; Zipeto, D.; Vita, C.; Sun, L.; Laurent, L.; Arenzana-Seisdedos, F.; Virelizier, J. L.; Michelson, S. Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. *J. Exp. Med.* **1998**, *188*, 855-866.
7. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
8. Randolph-Habecker, J.; Rahill, B.; Torok-Storb, B.; Vieira, J.; Kolattukudy, P. E.; Rovin, B. H.; Sedmak, D. D. The expression of the cytomegalovirus chemokine homolog US28 sequesters biologically active CC chemokines and alters IL-8 production. *Cytokine* **2002**, *29*, 37-46.
9. Casarosa, P; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R.; Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
10. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
11. Bais, C.; Santomasso, B.; Coso, O.; Arvanitakis, L.; Geas-Raaka, E.; Gutkind, J. S.; Asch, A. S.; Cesarman, E.; Gershengorn, M. C. Mesri, E. A. G-protein-coupled receptor of Kaposi's sarcoma associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature* **1998**, *391*, 86-89.
12. Holst, P. J.; Rosenkilde, M. M.; Manfra, D.; Chen, S.-C.; Wiekowski, M. T.; Holst, B.; Cifire, F.; Lipp, M.; Schwartz, T. W. Tumorigenesis induced by the HHV8-encoded chemokine receptor requires ligand modulation of high constitutive activity. *J. Clin. Invest.* **2001**, *108*, 1789-1796.
13. Maussang, D.; Verzijl, D.; van Walsum, M.; Leurs, R.; Holl, J.; Pleskoff, O.; Michel, D.; van Dongen, G. A. M. S.; Smit, M. J. Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13068-13073.
14. Golebiowski, A.; Klopfenstein, S. R.; Portlock, D. E. Lead compounds discovered from libraries. *Curr. Opin. Chem. Biol.* **2001**, 5, 273-284.
15. Golebiowski, A.; Klopfenstein, S. R.; Portlock, D. E. Lead compounds discovered from libraries: part 2. *Curr. Opin. Chem. Biol.* **2003**, *7*, 308-325.
16. Bleicher, K. H.; Böhm, H.-J.; Müller, K.; Alanine, A. I. Hit and lead generation: beyond high-troughput screening. *Nature Reviews Drug Discovery* **2003**, *2*, 369-378.
17. Keserű, G. M.; Makara, G. M. Hit discovery and hit-to-lead approaches. *Drug Discov. Today* **2006**, *11*, 741-748.
18. Hulshof, J.W.; de Esch, I. J. P.; Leurs, R. Synthesis and structure-Activity relationship of the first nonpeptidergic inverse agonists for the human cytomegalovirus encoded chemokine receptor US28. *J. Med. Chem.* **2005**, *48*, 6461-6471.
19. Hulshof J. W.; Vischer, H. F.; Verheij, M. H.; Fratantoni, S. A.; Smit, M. J.; de Esch, I. J. P.; Leurs, R. Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28. *Bioorg. Med. Chem.* **2006**, *14*, 7213-7230.
20. Schall, T. J.; McMaster, B. E.; Dairaghi, D. J. Modulators of US28. World (PTC) Patent WO0217900, **2002**.
21. McMaster, B. E.; Schall, T. J.; Penfold, M.; Wright, J. J.; Dairaghi, D. J. Bicyclic compounds as inhibitors of chemokine binding to US28. World (PTC) Patent WO03018549, **2003**.
22. Schall, T. J.; McMaster, B. E.; Dairaghi, D. J. Reagents and methods for the diagnosis of CMV dissemination. World (PTC) Patent WO0217969, **2002**.
23. Kenakin, T. P. Pharmacological proteus? *Trends Pharmacol. Sci.* **1995**, *16*, 256–258.
24. Kenakin, T. P. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.* **2001**, *15*, 598-611.

25. Brink, C. B.; Harvey, B. H.; Bodenstein, J.; Venter, D. P.; Oliver, D. W. Recent advances in drug action and therapeutics: relevance of novel concepts in G-protein-coupled receptor and signal transduction pharmacology. *Br. J. Clin. Pharmacol.* **2004**, 57, 373-387.
26. Berg, K. A.; Harvey, J. A.; Spampinato, U.; Clarke, W. P. Physiological relevance of constitutive activity of 5-HT2A and 5-HT2C receptors. *Trends Pharmacol. Sci.* **2005**, 26, 625-630.
27. Gbahou, F.; Rouleau, A.; Morisset, S.; Parmentier, R.; Crochet, S.; Lin, J. S.; Ligneau, X.; Tardivel-Lacombe, J.; Stark, H.; Schunack, W.; Ganellin, C. R.; Schwartz, J. C.; Arrang, J. M. Protean agonism at histamine H3 receptors in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11086–11091.
28. Baldi, E.; Bucherelli, C.; Schunack, W.; Cenni, G.; Blandina, P.; Passani, M. B. The H3 receptor protean agonist proxyfan enhances the expression of fear memory in the rat. *Neuropharmacol.* **2003**, *48*, 246–251.
29. Jansson, C. C.; Kukkonen, J. P.; Näsman, J.; Huifang, G.; Wurster, S.; Virtanen, R.; Savola, J.-M.; Cockcroft, V.; Åkerman, K. E. O. Protean agonism at alpha$_{2A}$-adrenoceptors. *Mol. Pharmacol.* **1998**, *53*, 963–968.
30. Pauwels, P. J.; Rauly, I.; Wurch, T.; Colpaert, F. C. Evidence for protean agonism of RX 831003 at alpha 2A-adrenoceptors by coexpression with different G alpha protein subunits. *Neuropharmacol.* **2002**, *42*, 855–863.
31. Yao, B. B.; Mukherjee, S.; Fan, Y.; Garrison, T. R.; Daza, A. V.; Grayson, G. K.; Hooker, B. A.; Dart, M. J.; Sullivan, J. P.; Meyer, M. D. In vitro pharmacological characterization of AM1241: a protean agonist at the cannabinoid CB_2 receptor? *Br. J. Pharmacol.* **2006**, *149*, 145-154.
32. Brink, C. B. Protean behavior by agonists: agonist directed trafficking of receptor signalling. *Trends Pharmacol. Sci.* **2002**, *23*, page 454.
33. Unangst, P. C.; Capiris, T.; Connor, D. T.; Doubleday, R.; Heffner, T. G.;MacKenzie, R. G.; Miller, S. R.; Pugsley, T. A.; Wise, L. D. (Aryloxy)alkylamines as selective human dopamine D4 receptor antagonists: potential antipsychotic agents. *J. Med. Chem.* **1997,** *40,* 4026-4029.
34. Liégeois, J.-F.; Eyrolles, L.; Ellenbroek, B. A.; Lejeune, C.; Carato, P.; Bruhwyler, J.; Géczy, J.; Damas, J.; Delarge, J. New pyridobenzodiazepine derivatives: modifications of the basic side chain differentially modulate binding to dopamine ($D_{4.2}$, D_{2L}) and serotonin (5-HT_{2A}) receptors, *J. Med. Chem.* **2002**, *45*, 5136-5149.
35. Farah, A. Atypicality of atypical antipsychotics. *Prim. Care Companion J. Clin. Psychiatry* **2005**, 7, 268-274.
36. Smits, R. A.; Lim, H. D.; Stegink, B.; Bakker, R. A. de Esch, I. J.; Leurs, R. Characterization of the histamine H4 receptor binding site. Part 1. Synthesis and pharmacological evaluation of dibenzodiazepine derivatives. *J. Med. Chem.* **2006,** *49*, 4512-4516.
37. Mouithys-Mickalad, A.; Kauffmann, J.-M.; Petit, C.; Bruhwyler, J.; Liao, Y.; Wikström, H.; Damas, J.; Delarge, J.; Deby-Dupont, G.; Géczy, J.; Liégeois, J.-F. Electrooxidation potential as a tool in the early screening for new safer clozapine-like analogues. *J. Med. Chem.* **2001**, *44*, 769 -776.
38. van der Stelt, C.; Funcke, A. B.; Tersteege, H. M.; Nauta, W. T. The effect of alkyl substitution in drugs. *Arzneim. Forsch.* **1966**, *16*, 1342-1345.
39. van der Stelt, C. Tetrahydrodibenzozocinen, hun zuur-additiezouten en werkwijzen ter bereiding van deze verbindingen. Patent BE616983, **1961**.
40. van der Stelt, C.; Heus, W. J.; Nauta, W. T. The synthesis of some N-substituted dibenz[*b*,*f*]azocine derivatives. *Arzneim. Forsch.* **1964**, *14*, 116-117.
41. Gootjes, J.; Funcke, A. B. H.; Nauta, W. T. Synthesis and pharmacology of a number of seco analogues of 2-(*p*-chlorophenyl)-1,3,4,6,7,11*b*-hexahydro-9,10-dimethoxy-2*H*-benzo[*a*]-quinolizine. *Arzneim. Forsch.* **1967**, *17*, 1145-1149.

Chapter 6

Identification of a molecular determinant that leads to neutral antagonism or inverse agonism on US28

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Abstract

Many viruses encode constitutively active G protein-coupled receptors to alter intracellular signaling pathways in the host, thereby changing the normal cellular programming. Blocking these viral-encoded receptors and inhibiting their constitutive signaling could be a new strategy for the development of therapeutics against viral-induced pathologies. Currently, the human cytomegalovirus encoded receptor US28 is the only viral-encoded GPCR for which small non-peptide inhibitors have been identified. In this study, we describe the synthesis and pharmacological characterization of novel inverse agonists acting on US28. Moreover, we report the discovery of a neutral antagonist for the viral-encoded receptor and we show how we can modulate the functional activity of this molecule by the introduction of small structural modifications. These ligands with different functional activities can help us to investigate the importance of US28 signaling in viral pathogenesis and in the future these molecules may serve as putative leads for innovative anti-viral intervention.

Introduction

Large DNA viruses, e.g. herpes- and poxviruses, have genes that encode G protein-coupled receptors that help to exploit the host cell for viral reproduction and survival.[1,2] These viral genes may have been pirated from the host genome during the long co-evolution of virus and host as they bear great resemblance to the cellular host proteins in structure and/or function. Interestingly, most viral-encoded GPCRs show a high sequence identity with human chemokine receptors, but compared to their mammalian homologues many of these viral-encoded chemokine receptors activate different signaling pathways in a ligand-independent manner.[3-5] More and more information about vGPCRS with regards to their signal transduction pathways and chemokine binding properties has been acquired, but the exact role of these receptors during the viral life cycle and viral pathogenesis has not been elucidated yet. However, increasing evidence suggests that they might play an essential role in virus-associated diseases and may therefore represent interesting and challenging new drug targets for a novel class of anti-viral therapy.[4]

One of these virally encoded GPCRs is the human cytomegalovirus (HCMV) encoded constitutively active receptor US28.[6] HCMV is highly species-specific β-herpesvirus that persists lifelong in the host in a latent form without any clinical symptoms. However, primary infection or reactivation of the virus in immunocompromised hosts, such as premature neonates, transplant recipients or acquired immunodeficiency syndrome (AIDS) patients, can cause severe and even fatal disorders.[7,8]

Infection with HCMV is suggested to be associated with vascular diseases,[9-11] inflammatory and autoimmune diseases as well as cancer.[12] Interestingly, US28 is proposed to have an important role in the HCMV-mediated development of vascular diseases due to the US28-induced migration of smooth muscle cells after infection. This migration towards the vascular intima is a typical characteristic of the formation of atherosclerotic and restenotic lesions.[13] In people infected with human immunodeficiency virus (HIV), co-infection with HCMV has been shown to cause an increased risk of disease progression to AIDS and dementia.[14] US28 is also suggested to play a role in the relationship between HCMV and HIV, because the vGPCR can act as a co-receptor for HIV-1

entry into cells in vitro.[15] Moreover, a potential symbiotic relationship between HCMV and HIV-1 has been suggested, because HIV-1 can establish the immunosuppression needed for the emergence of HCMV from latency and maximal HCVM replication, while HCMV facilitates HIV-1 infection through US28 and provides an additional mechanism for cell entry by HIV-1.[16]

US28 can bind several inflammatory CC chemokines, such as CCL2, CCL3, CCL4, CCL5, and CCL7, as well as the only member of the CX3C chemokine family, namely CX3CL1, with high affinity.[17-20] This large spectrum chemokine binding profile suggests that US28 could act as a chemokine scavenger by removing the CC chemokines from the extracellular environment, thereby helping to overturn the immune system of the host.[21,22] Moreover, US28 constitutively activates different signaling pathways, including phospholipase C, NF-κB, cAMP-response element binding protein (CREB) and nuclear factor activated T cell (NFAT). Via these pathways the virus alters the normal homeostasis of the cell for its own benefit. [5,23-25] In view of the potential role of US28 during viral infection, small molecules that inhibit the basal signaling of the receptor and block chemokine or HIV binding to US28 are considered promising therapeutics against HCMV-mediated disorders.[5]

Currently, US28 is the only viral-encoded GPCR for which small nonpeptidergic ligands have been identified. Several molecules that inhibit chemokine binding to US28 have been reported in the patent literature, e.g. the non-selective 5-hydroxytryptamine (5-HT) receptor antagonist methiothepin was reported to bind to US28 in a reversible manner with an IC_{50} value of 0.3 μM. Moreover, this compound was shown to act as an agonist in cytoplasmic calcium mobilization experiments by inducing a rise in intracellular Ca^{2+}.[26] Next to this, we identified VUF2274 (**1**) as the very first inverse agonist acting on a virally encoded GPCR (Figure 1), i.e. this molecule is able to completely block the constitutive signaling of the receptor. Moreover, it has been shown that compound **1** inhibits the US28-mediated HIV-1 entry in cells.[27] Recently, the synthesis of an extended series of analogues of compound **1** was reported and the first structure-activity relationships for inverse agonism on a viral-encoded chemokine receptor were elucidated.[28,29]

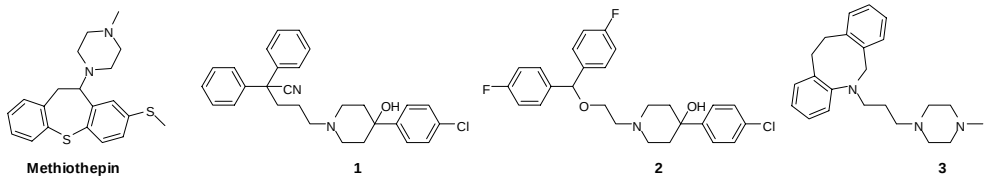


Figure 1. Chemical structure of methiothepin and compound **1**-**3**.

In our search for novel ligands acting on US28, a large number of compounds from our in-house compound collection was screened for their binding affinity and inverse agonistic properties (Chapter 5). Several interesting ligands with different pharmacological profiles have been found, such as the novel inverse agonist **2** and the very first nonpeptidergic neutral antagonist acting on US28, namely compound **3**.

Many GPCRs exist in one of two main conformers of the receptor, namely the inactive state R or the active state R* (Figure 2).[30] The active state is considered to be responsible for G protein activation, while the inactive state is not coupled to G proteins. The equilibrium between the active and the inactive state determines the level of the basal signaling.[31-33] Agonists are molecules that bind to the receptor and stabilize or increase the amount of receptors in the active state. Inverse agonists are able to reduce the constitutive basal signaling of a receptor by stabilizing or enriching the number of receptors in the inactive state.[34] In contrast, neutral antagonists are able to bind to the receptor without altering the equilibrium between the active and inactive states of the receptor, and by doing so, having no influence on the basal singaling. These antagonists are important tools for ligand classification, because compounds with these characteristics that competitively bind to the receptor as well, will act as functional antagonists to compounds with agonistic or inverse agonistic properties.[35]

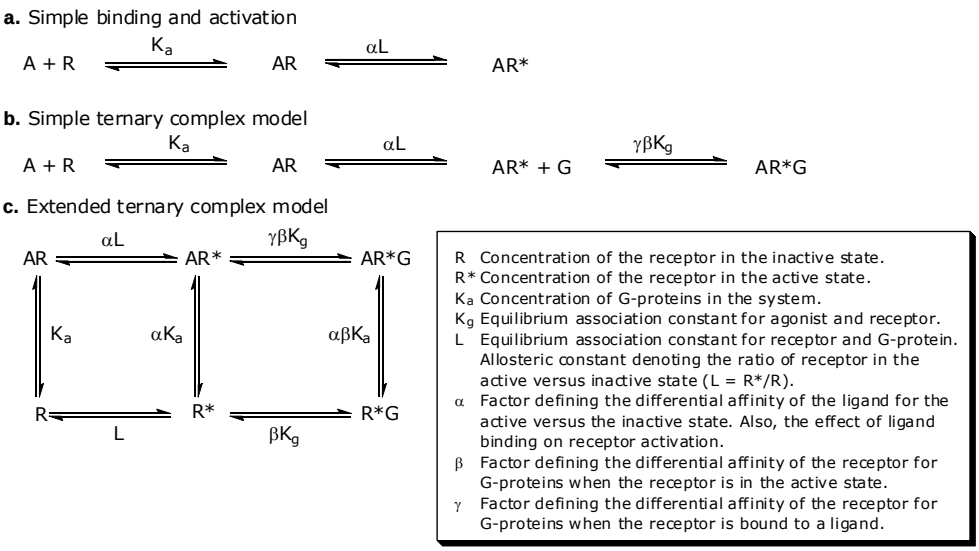


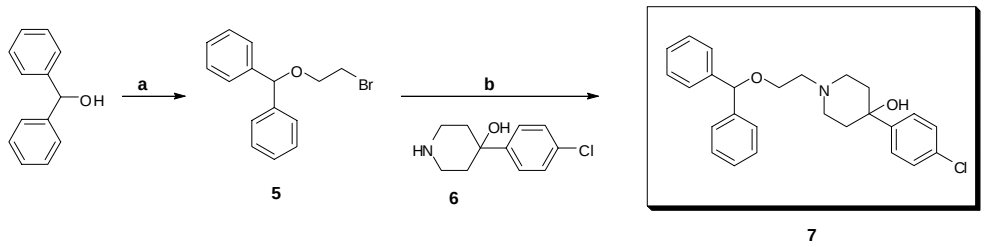
Figure 2. Three simple models for GPCR systems. a. The classical view of receptor binding and activation shows agonist (A) binding to an inactive (R) receptor to form a complex (AR). This complex isomerizes the receptor to the active state (R*) due to the efficacy of the agonist. b. Activation of the receptor by an agonist (A) is followed by topographically distinct binding of the active receptor to the G protein (G). The response follows from the ternary AR*G complex. c. The extended ternary complex permits allows the for the spontaneous formation of an active-state receptor (R*) in absence of an agonist. The active receptor R* can interact with, and activate, a G protein. Activation of the receptor from R to R*, which can happen spontaneously or through ligand binding, modifies the affinity of the receptor for the G protein by the factors β and γ. Adapted from Kenakin et al.[36]

In this study, we describe the synthesis and pharmacological characterization of several analogues of compounds **2** and **3** to investigate the structure-activity relationships of these ligands. Moreover, we show how the synthesis of hybrid compound **4a**, which contains structural features of both inverse agonist **1** and neutral antagonist **3**, resulted in the discovery of a molecular determinant that leads to neutral antagonism or inverse agonism for US28. Thus, the functional activity of ligands on US28 can be modulated by subtle structural changes. These insights will enable the design of molecules with different efficacies to investigate the significance of US28 signaling in viral pathogenesis.

Chemistry

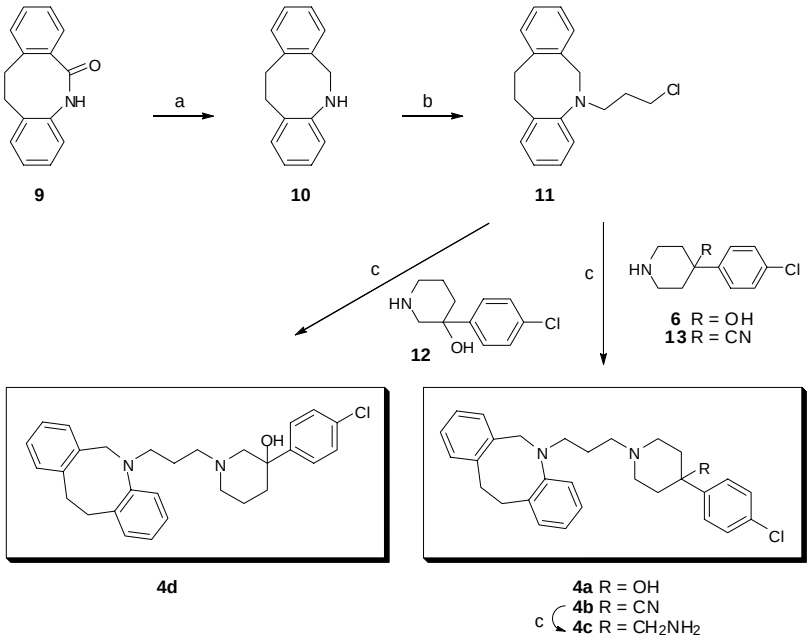
Bromide intermediate **5**, which was synthesized in a quantitative yield by an acid-catalysed condensation of diphenylmethanol with 2-bromoethanol with azeotropic removal of water,[37] was reacted with 4-(4-chlorophenyl)piperidin-4-ol **6** in the microwave in the presence of NaI, Na_2CO_3 and CH_3CN to give compound

7 (Scheme 1). Compound **8** (Table 1) was synthesized in an analogue manner by the alkylation of the commercially available 4,4'-(4-chlorobutane-1,1-diyl)*bis*(fluorobenzene) with piperidine **6**.



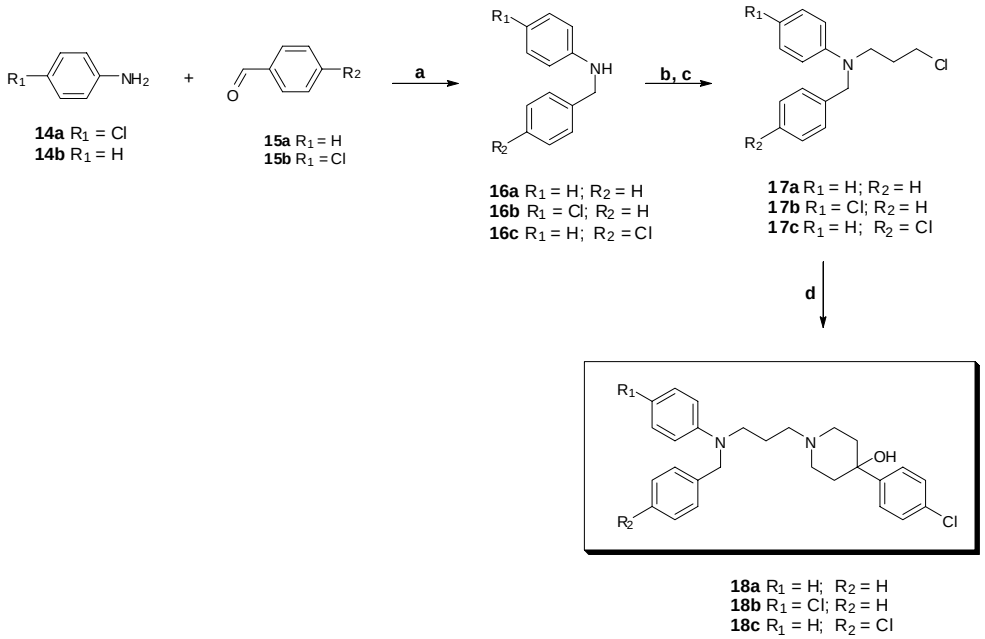
Scheme 1. Synthetic pathway for the synthesis of compound **7**. Reagents and conditions: (a) *p*-TSA, 2-bromoethanol, toluene, reflux; (b) NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

The synthesis of compounds **4a**-**4d** is outlined in Scheme 2.



Scheme 2. Synthetic pathway for the synthesis of **4a**-**4d**. Reagents and conditions: (a) $LiAlH_4$, THF, reflux; (b) 1-bromo-3-chloropropane, Na_2CO_3, microwave (15 min, 200 °C); (c) NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C) or NaI, Na_2CO_3, CH_3CN, reflux; (d) $AlCl_3$, $LiAlH_4$, THF.

Reduction[38] of 11,12-dihydrodibenzo[*b*,*f*]azocin-6(5*H*)-one **9** with $LiAlH_4$ resulted in 5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine **10**, which was alkylated with 1-bromo-3-chloropropane in the microwave (15 min, 200 °C) to yield intermediate **11**. *N*-alkylation of piperidine moieties **6**, **12** and **13** with chloride **11** afforded target compounds **4a**, **4d** and intermediate **4b** respectively. The 3-substituted piperidine moiety **12** was synthesized by a Grignard reaction of BOC-protected piperidin-3-one with 4-chlorophenyl magnesium bromide followed by a deprotection under acidic conditions.[39] Piperidine moiety **13** was synthesized starting from *bis*(2-chloroethyl)amine hydrochloride as previously described in the literature.[29,40] The nitrile group of intermediate **4b** was reduced in the presence of $AlCl_3$ and $LiAlH_4$ to yield target compound **4c**.

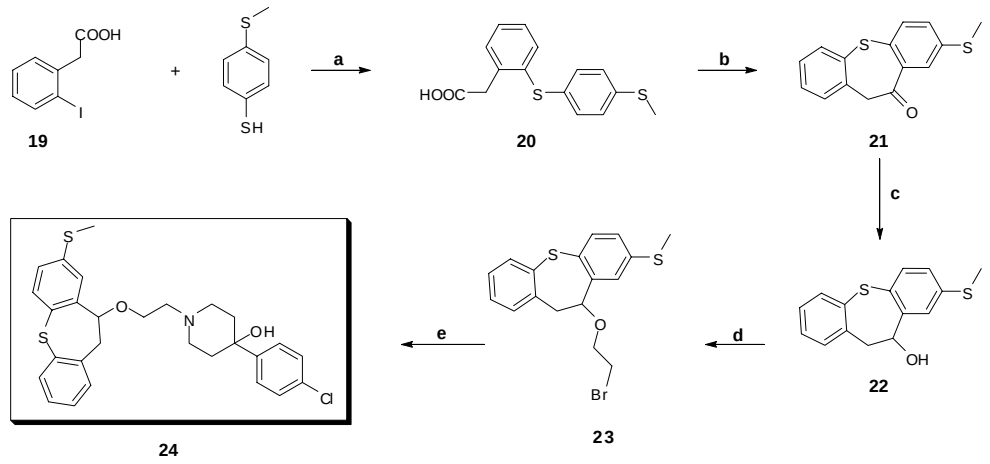


Scheme 3. Synthetic pathway for the synthesis of **18a**-**18c**. Reagents and conditions: (a) $NaBH(OAc)_3$, CH_3COOH, DCE; (b) $NaNH_2$, toluene, reflux; (c) 1-bromo-3-chloropropane, reflux; (d) **6**, NaI, Na_2CO_3, CH_3CN, microwave (15 min, 160 °C).

Amines **16a**-**c** were deprotonated with $NaNH_2$ in toluene at reflux temperature followed by an alkylation with 1-bromo-3-chloropropane to give intermediates **17a**-**c**, which were reacted with piperidine **6** in the presence of NaI, Na_2CO_3, and CH_3CN (Scheme 3). Unsubstituted amine **16a** was commercially available, and

amines **16b** and **16c**, which were used for the synthesis of compounds **18b** and **18c**, were synthesized in a quantitative yield via a reductive amination reaction with the corresponding anilines **14a**-**b** and benzaldehydes **15a**-**b** in the presence of sodium triacetoxyborohydride and acetic acid in DCE.

Rigid tricyclic analogue **24** was synthesized as depicted in Scheme 4. Intermediate **20** was synthesized by the reaction of 2-(2-iodophenyl)acetic acid **19** with 4-(methylthio)benzenethiol in the presence of KOH, Cu and water.[41] The ring closure to the tricyclic system of **21** was performed by a Friedel-Crafts acylation of **20** with PPA in toluene.[42] Reduction of the carbonyl group of **21** with $NaBH_4$ in MeOH resulted in alcohol **22**, which was reacted with $BF_3{\cdot}(Et)_2O$ and 2-bromoethanol in toluene to give bromide **23**.[43] *N*-akylation of piperidine **6** with **23** yielded target compound **24**.



Scheme 4. Synthetic pathway for the synthesis of **24**. Reagents and conditions: (a) Cu, KOH, water, reflux; (b) PPA, toluene, reflux; (c) $NaBH_4$, MeOH; (d) $BF_3{\cdot}(Et)_2O$, 2-bromoethanol, toluene; (e) **6**, NaI, Na_2CO_3, CH_3CN, reflux.

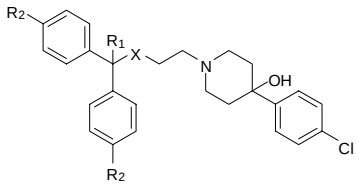
Results and discussion

Screening of our in-house compound collection (Chapter 5) revealed two interesting novel hits, namely L118 (**2**) and A194 (**3**). In this study, both compounds were able to displace more than 50% of [^{125}I]CCL5 from US28 at a concentration of 10 μM. Both compounds were re-screened by evaluating their ability to dose-dependently displace [^{125}I]CCL5 binding to US28 and by

determining their inverse agonistic properties by investigating their potential to inhibit the constitutive inositol phosphate production in US28-expressing SVEC4-10 cells.

Compounds **2** and **3** were able to bind to US28 with IC_{50} values of 2.3 and 4.9 μM, respectively. Interestingly, compound **2** acts as an inverse agonist on US28 by inhibiting the ligand-independent signaling of the receptor. In contrast, compound **3** did not influence the basal signaling of the receptor, thereby acting as a neutral antagonist. This is the very first report of a small nonpeptidergic molecule acting as a neutral antagonist on a viral-encoded receptor, namely US28. To further investigate a possible correlation between the structures of the ligands and their functional activity on US28, several analogues of **2** and **3** were obtained from our in-house database or synthesized. All compounds were evaluated for their binding properties by their ability to displace [^{125}I]CCL5 binding to US28 in a dose-dependent manner. The inverse agonistic properties were determined for a selection of compounds.

Table 1. Chemical structures and pharmacological properties of compounds **1**, **2**, **7** and **8** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	Code	R_1	R_2	X	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1	VUF2274	CN	H	CH_2	4.9 (4.4 - 5.5)	6.3 (5.9 – 6.8)
2	L118	H	F	O	2.3 (1.5 – 3.2)	4.6 (2.5 - 4.0)
7	VUF6999	H	H	O	2.1 (1.8 – 2.4)	3.5 (1.7 – 5.4)
8	VUF6968	H	F	CH_2	3.0 (2.5 – 3.5)	5.2 (3.2 – 7.2)

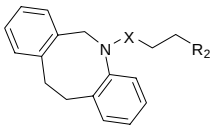
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

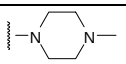
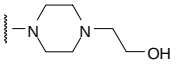
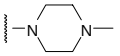
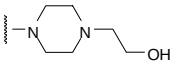
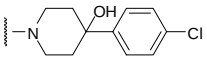
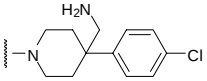
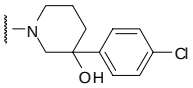
*Structural analogues of L118 (**2**)*

To study the importance of the fluoro atoms in the phenyl rings of **2** and the oxygen atom in the linker between the diphenyl group and the piperidine moiety in this molecule, compounds **7** and **8** were synthesized (Table 1). In our

previous study[28] it was demonstrated that chloro substitution at the para position of both rings of the diphenyl group resulted in a decrease of affinity compared to reference compound **1**. However, the introduction of the two fluoro atoms in **8** causes an increase in affinity compared to lead compound **1** and an affinity comparable to that of compound **2**. Moreover, removal of the fluoro atoms, resulting in compound **7**, does not change affinity or efficacy on US28.

Table 2. Chemical structures and pharmacological properties of compounds **3**, **4a**, **4c**-**d** and **25-27** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	Code	X	R_1	IC_{50} (μM)[a]	EC_{50} (μM)[b]
3	A194	CH_2		4.9 (2.8 – 7.1)	3% ± 15
25	B82	CH_2		1.6 (1.3 – 1.9)	0% ± 6
26	A190	C(=O)		> 100	0% ± 16
27	B78	C(=O)		> 100	0% ± 5
4a	VUF10000	CH_2		0.7 (0.6 – 0.9)	4.7 (2.8 – 6.6)
4c	VUF10253	CH_2		1.6 (1.0 – 2.2)	7.6 (4.8 – 10.5)
4d	VUF10347	CH_2		0.9 (0.7 – 1.0)	3.9 (3.6 – 4.2)

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

*Structural analogues of A194 (**3**)*

To investigate the effect of the structure on the functional activity of the receptor, analogues of compound **3** were evaluated on US28 (Table 2 and 3). Firstly, several molecules were taken from our in-house library and pharmacologically analyzed (compounds **25**-**27**). Replacement of the *N*-methyl group of **3** by an ethanol group in **25** results in a 3-fold increase in affinity, but the antagonistic properties are maintained. Interestingly, changing the basic properties of the nitrogen atom of both compounds **3** and **25** by the introduction

of an amide group in compounds **26** and **27** causes a significance decrease in binding affinity.

It was previously discovered that a 4-phenylpiperidine moiety is essential for inverse agonism on US28.[28] Thus, the *N*-methylpiperazine group of **3** was replaced by the 4-(4-chlorophenyl)piperidin-4-ol moiety of lead compound **1**, resulting in compound **4a**, leading to the anticipated recovery of the inverse agonistic properties (Figure 3). Intriguingly, this structural modifcation has a great influence on the functional activity of the ligands and reveals a molecular determinant that leads to neutral antagonism or inverse agonism. Moreover, hybrid compound **4a** has a 7-fold higher binding affinity than that of lead compound **1** or neutral antagonist **3**.

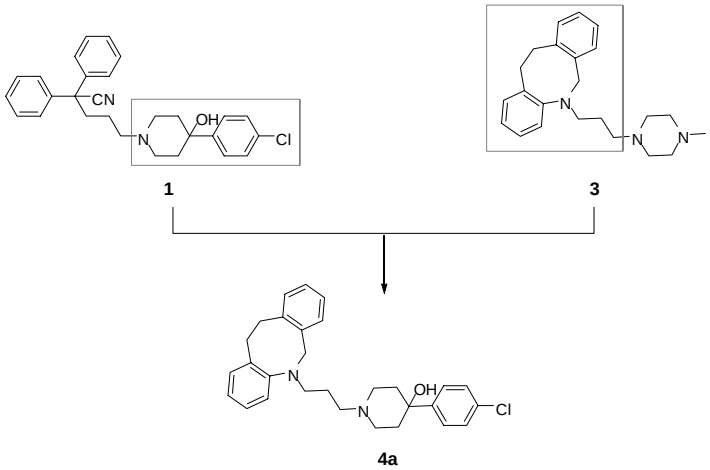


Figure 3. Chemical structures of inverse agonist **1**, neutral antagonist **3** and hybrid compound **4a**.

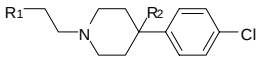
It is known for many GPCRs that small structural modifications can cause dramatic changes in the functional activities on the receptor.[44,45] It is shown for constitutively active receptors that inverse agonists can activate molecular switches that are essential to give a conformational rearrangement that leads to the inverse agonistic effect of these ligands.[34,46] Apparently, the structures of compounds **3** and **25** cannot induce the conformational change necessary to stabilize or enrich the number of receptors in the inactive state. Thus, these compounds do not modulate the basal signaling of the receptor, thereby acting

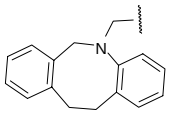
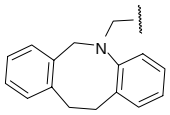
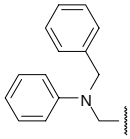
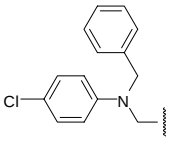
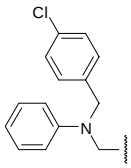
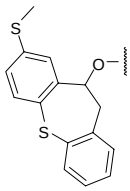
as neutral antagonists. In contrast, the structure of compound **4a** is able to induce the conformational change to inactivate the receptor, thereby inhibiting the basal signaling of the receptor. Thus, the cyclic amine group attached to the propyl linker can be considered as an interesting molecular determinant for the modulation of the functional activity of ligands acting on US28.

Previously, it was shown that a methylamine group at the 4-position of the piperidine ring resulted in a 6-fold increase in binding affinity compared to lead compound **1** (Chapter 3).[29] Thus, the 4-hydroxy group of compound **4a** was replaced by a methylamine group, resulting in compound **4c**. However, this structural modification did not cause an increase in the binding affinity and efficacy on US28. Introduction of a chiral center by moving the hydroxy group and the 4-chlorophenyl substituent to the 3-position of the piperidine ring, as in compound **4d**, did not improve the binding affinity and efficacy on US28 either.

To investigate the significance of the rigid tricyclic structure of compound **4a** several flexible analogues were synthesized (Table 3). The more rigid system in compound **4a** proofs to be of importance for affinity, because removal of the ethylene bridge between the two phenyl rings as in open analogue **18a** causes a more than 10-fold decrease in binding affinity. Introduction of a chloro atom at the 4-position of one of the phenyl rings, resulting in compounds **18b** and **18c**, shows an interesting regioselectivity. The efficacies of both compounds are comparable, but the position of the chloro atom in compound **18b** was preferred, resulting in a 3-fold higher binding affinity compared to compound **18c**. To investigate the influence of the tricyclic system of methiothepin, compound **24** was synthesized. Unfortunately, this compound shows an almost 18-fold decrease of binding affinity compared to compound **4a**.

Table 3. Chemical structures and pharmacological properties of compounds **4a**, **4c**, **18a**-**18c** and **24** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	Code	R_1	R_2	IC_{50} (μM)[a]	EC_{50} (μM)[b]
4a	VUF10000		OH	0.7 (0.6 – 0.9)	4.7 (2.8 – 6.6)
4c	VUF10253		CH_2NH_2	1.6 (1.0 – 2.2)	7.6 (4.8 – 10.5)
18a	VUF10254		OH	4.1 (2.8 – 5.4)	3.0 (2.8 – 6.0)
18b	VUF10257		OH	1.8 (1.4 – 2.1)	5.0 (3.9 – 6.2)
18c	VUF10258		OH	5.7 (4.3 – 7.1)	5.5 (5.0 – 6.0)
24	VUF10255		OH	12.4 (11.2 – 13.5)	7.1 (4.9 – 9.3)

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

Conclusions

Screening of a selection of compounds from our in-house database resulted in the identification of compound **2** and **3** as new starting points for hit optimization programs. While compound **2** was shown to have inverse agonistic properties on US28, molecule **3** was identified as the very first neutral antagonist acting on a viral-encoded receptor, namely US28. In this study, we report the synthesis and SAR of a series of analogues of compounds **2** and **3** and

we studied the effect of the structure of the ligands on the functional activity on the receptor. Remarkably, replacement of the piperazine moiety of compound **3** by a 4-(4-chlorophenyl)piperidin-4-ol group as in compound **4a** resulted in complete recovery of the inverse agonistic properties. Thus, the nature of the nitrogen containing ring system attached to the propyl chain can be considered as a molecular determinant for the regulation of the functional activity of this series of ligands. Antagonists are important tools to study receptor pharmacology, because these ligands act as functional antagonists to compounds with agonistic or inverse agonistic properties.

Experimental section

General procedures.

THF and DCM were freshly distilled from lithium aluminium hydride. All reactions were performed under an atmosphere of dry nitrogen. Microwave reactions were performed in a CEM Explorer single mode MW reactor equipped with auto sampler. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer unless otherwise stated. J.T. Baker silica gel was used for flash chromatography. HRMS mass spectra were recorded on a Finnigan MAT 900 mass spectrometer. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV-VIS detector set at 254 nm, with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph mass spectrometer. The analyses were performed using the following conditions; condition I: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 70% MeOH - 30% H_2O-0.1% formic acid (Method Ia); 60% MeOH - 40% H_2O-0.1% formic acid (Method Ib) or 50% MeOH - 50% H_2O-0.1% formic acid (Method Ic). Flow rate = 1.0 mL/min. Total run time 15 min unless otherwise stated. Condition II: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 50% CH_3CN - 50% H_2O-0.1% formic acid (Method IIa); 40% CH_3CN - 60% H_2O-0.1% formic acid (Method IIb), 35% CH_3CN - 65% H_2O-0.1% formic acid (Method IIc), 30% CH_3CN - 70% H_2O-0.1% formic acid (Method IId) or 25% CH_3CN - 75% H_2O-0.1% formic acid (Method IIe). Flow rate = 1.0 mL/min. Total run time 20 min. Compounds that were isolated as fumaric acid salts all showed an extra peak around two minutes. Fumaric acid blancs were used to determine the *t*R of fumaric acid. Purities calculated are based on RP HPLC-UV peak surface area of the compounds (disregarding the fumaric acid peak). Compounds **2**, **3** and **25-27** were taken from our in-house database.

1-(2-(*bis*(4-Fluorophenyl)methoxy)ethyl)-4-(4-chlorophenyl)-piperidin-4-ol maleate (2). This compound was obtained from our in-house database. ^{1}H NMR ($CDCl_3$): δ 1.80-2.01 (m, 2H), 2.32-2.55 (m, 2H), 3.16-3.56 (m, 7H), 3.82 (t, *J* = 4.8 Hz, 2H), 5.32 (s, 1H), 6.16 (s, 2H), 6.92-7.00

(m, 4H), 7.19-7.39 (m, 10H). ^{13}C NMR (MeOH-d_4): δ 36.23, 48.60, 57.24, 63.76, 69.12, 84.13, 116.14, 116.57, 127.48, 129.54, 129.97, 130.14, 134.29, 136.72, 138.65, 138.71, 146.97, 161.34, 166.21, 170.89. Anal. RP-HPLC *Ib*: t_R = 9.63 min (purity 99%), *IIa*: t_R = 8.18 min (purity 100%), *IIb* (total run time 30 min): t_R = 21.49 min (purity 98%). HRMS (ESI) m/z calcd for $C_{27}H_{28}ClF_2NO$: 457.1620; found: 457.1607.

5-(3-(4-Methylpiperazin-1-yl)propyl)-5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine trihydrochloride (3). This compound was obtained from our in-house database. ^{1}H NMR (MeOH-d_4): δ 2.08-2.33 (m, 2H), 3.00 (s, 3H), 3.13-3.79 (m, 14H), 3.31 (s, 2H), 3.87 (t, *J* = 8.1 Hz, 2H), 6.99 (d, *J* = 4.0 Hz, 1H), 7.10-7.41 (m, 7H). ^{13}C NMR (MeOH-d_4, 400 MHz): δ 22.45, 32.47, 34.57, 43.35, 50.03, 51.75, 54.89, 55.85, 61.00, 125.20, 127.89, 128.84, 130.90, 130.96, 131.65, 132.72, 133.83, 136.01, 138.00, 142.10. Anal. RP-HPLC *Ic*: t_R = 6.84 min (purity 100%), *IIe*: t_R = 6.14 min (purity 97%). HRMS (EI) m/z calcd for $C_{23}H_{31}N_3$: 349.2518; found: 349.2506.

General method A. 1-(2-(Benzhydryloxy)ethyl)-4-(4-chlorophenyl)piperidin-4-ol fumarate (7). 5 (0.58 g, 1.99 mmol), which was synthesized following a method previously described,[37] 4-(4-chlorophenyl)piperidin-4-ol **6** (0.51 g, 2.41 mmol), NaI (0.30 g, 2.00 mmol), Na_2CO_3 (0.42 g, 3.96 mmol) and 3 mL CH_3CN were added in a 10 mL microwave vessel and this was reacted during 15 minutes in the microwave at a temperature of 160 °C (settings: ramp time 5 min, hold time 15 min, power 200 watt, pressure 17.2 bar). The solvent was removed in vacuo and the residue was diluted with water (20 mL), followed by an extraction with DCM (3 x 15 mL). The combined organic layers were washed with water (3 x 40 mL) and brine (40 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (0-50% EtOAc in DCM) gave 480 mg (57%) of the free base as an oil. This was dissolved in EtOAc and acidified by the addition of a saturated solution of fumaric acid in Et_2O. The fumaric salt was isolated by filtration and recrystallized from MeOH/Et_2O to give 281 mg (52%) of **7** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.60-1.81 (m, 2H), 2.08-2.43 (m, 2H), 2.84-3.25 (m, 6H), 3.65-3.78 (m, 2H), 5.28 (s, 1H), 6.63 (s, 2H), 7.11-7.36 (m, 14H). ^{13}C ($CDCl_3$): δ 36.29, 49.42, 56.91, 64.68, 69.54, 84.64, 126.57, 127.30, 128.09, 128.89, 134.06, 135.30, 141.78, 148.74, 169.53. Anal. RP-HPLC *Ib*: t_R = 8.60 min (purity 100%), *IId*: t_R = 12.13 min (purity 100%). HRMS (ESI) m/z calcd for $C_{26}H_{28}ClNO_2$: 421.1808; found: 421.1803.

General method B. 1-(4,4-*bis*(4-Fluorophenyl)butyl)-4-(4-chlorophenyl)piperidin-4-ol (8). A solution of 4,4'-(4-chlorobutane-1,1-diyl)*bis*(fluorobenzene) (0.56 g, 1.99 mmol), 4-(4-chlorophenyl)piperidin-4-ol **6** (0.51 g, 2.41 mmol), NaI (0.30 g, 2.00 mmol) and Na_2CO_3 (0.43 g, 4.06 mmol) in CH_3CN (20 mL) was refluxed overnight. The solvent was removed in vacuo, the residue was diluted with water (50 mL) and extracted with DCM (3 x 30 mL). The combined organic layers were washed with water (3 x 50 mL) and brine (50 mL), dried over anhydrous $MgSO_4$, filtered and

evaporated in vacuo. Purification by flash chromatography (0-100% EtOAc in DCM) and recrystallization from EtOAc gave 853 mg (94%) of **8** as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.48-1.72 (m, 5H), 1.92-2.24 (m, 4H), 2.30-2.55 (m, 4H), 2.69-2.85 (m, 2H), 3.86 (t, *J* = 7.8 Hz, 1H), 6.90-7.43 (m, 12H). ^{13}C ($CDCl_3$): δ 25.76, 34.27, 38.71, 49.83, 50.14, 58.92, 71.41, 115.57, 115.78, 126.47, 128.82, 129.43, 129.51, 133.22, 140.86, 140.89, 147.16, 160.54, 162.97. Anal. RP-HPLC *Ib*: t_R = 8.78 min (purity 100%), *IIb*: t_R = 13.99 min (purity 100%). HRMS (EI) m/z calcd for $C_{27}H_{28}ClF_2NO$: 455.1827; found: 455.1826.

General method C. 1-(3-((4-chlorobenzyl)(phenyl)amino)-propyl)-4-(4-chlorophenyl)-piperidin-4-ol fumarate (18c). (i) A solution of aniline **14b** (1.82 mL, 20.0 mmol), 4-chlorobenzaldehyde **15b** (2.35 mL, 20.0 mmol) and CH_3COOH (1.14 mL, 19.9 mmol) in DCE (100 mL) was stirred for 24h at room temperature and $NaBH(OAc)_3$ (6.36 g, 30.0 mmol) was added. The reaction mixture was stirred for another 18h, quenched with 5% Na_2CO_3 (100 mL) and the water layer was extracted with DCM (3 x 100 mL). The combined organic layers were washed with brine (150 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 4.42 g (100%) of **16c** as a brown solid. ^{1}H NMR ($CDCl_3$): δ 4.06 (br s, 1H), 4.29 (s. 2H), 6.51-6.58 (m, 2H), 7.07-7.14 (m, 2H), 7.24-7.36 (m, 5H).

(ii) $NaNH_2$ (0.39 g, 10.0 mmol) was added to a solution of **16c** (1.09 g, 5.01 mmol) in toluene (15 mL) and this was refluxed for 18h. 1-Bromo-3-chloropropane (2.5 mL, 25.3 mmol) was added and the reaction mixture was refluxed for another 18h. Water (25 mL) was added and the water layer was extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with water (3 x 30 mL) and brine (40 mL), dried over anhydrous Na_2SO and filtered. After evaporation under reduced pressure, the residue was purified by flash chromatography (5% DCM in hexane) to give 142 mg (10%) of **17c** as a light yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.98-2.18 (m, 2H), 3.45-3.68 (m, 4H), 4.50 (s, 2H), 6.51-6.75 (m, 3H), 7.03-7.28 (m, 6H).

(iii) Following method B using **17c** gave 142 mg of the free base as an oil. This was converted to the fumaric salt as described for **7** to give 178 mg (63%) of **18c** as a white solid. ^{1}H NMR (MeOH-d_4): δ 1.81-1.93 (m, 2H), 1.97-2.17 (m, 2H), 2.19-2.39 (m, 2H), 2.98-3.39 (m, 6H), 3.50 (t, *J* = 7.2 Hz, 2H), 4.54 (s, 2H), 6.59-6.30 (m, 4H), 7.02-7.51 (m, 11H).^{13}C (MeOH-d_4): δ 23.81, 36.88, 49.57, 50.09, 55.21, 55.84, 69.75, 114.39, 118.29, 127.48, 129.44, 129.58, 129.61, 130.30, 133.52, 134.07, 137.00, 139.26, 147.47, 149.37, 173.62. Anal. RP-HPLC *Ib*: t_R = 4.87 min (purity 100%), *IIb*: t_R = 3.89 min (purity 100%). HRMS (EI) m/z calcd for $C_{27}H_{30}Cl_2N_2O$: 468.1735; found: 468.1721.

4-(4-chlorophenyl)-1-(3-(11,12-dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)propyl)piperidine-4-ol fumarate (4a). Following method A using **11** (0.29 g, 1.01 mmol) gave 289 mg of the free base as an oil. This was converted to the fumaric salt as described for **7** and recrystallized from MeOH/Et_2O

to give 199 mg (38%) of **4a** as white crystals. ^{1}H NMR ($CDCl_3$): δ 1.50-1.92 (m, 5H), 2.09-2.36 (m, 2H), 2.41-2.70 (m, 4H), 2.78-3.34 (m, 8H), 4.11 (s, 2H), 6.69 (s, 2H), 6.72-7.35 (m, 12H). ^{13}C ($CDCl_3$): δ 23.15, 33.28, 34.22, 35.92, 48.11, 51.14, 55.08, 60.96, 69.57, 119.32, 122.17, 125.91, 126.82, 127.15, 128.39, 128.79, 129.73, 131.08, 132.94, 135.45, 136.06, 137.65, 141.83, 145.37, 150.02, 171.33. Anal. RP-HPLC *Ib* (total run time 20 min): t_R = 14.43 min (purity 100%), *IIc*: t_R = 11.35 min (purity 97%). HRMS (EI) m/z calcd for $C_{29}H_{33}ClN_2O$: 460.2281; found: 460.2284.

(4-(4-chlorophenyl)-1-(3-(11,12-dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)propyl)piperidin-4-yl)methanamine fumarate (4c). (i) Following method B using **11** and 4-(4-chlorophenyl)-piperidine-4-carbonitrile hydrochloride **13**, which was synthesized as previously described in the literature,[29] afforded 675 mg of **4b** as a light yellow oil. The crude product was used without further purification.

(ii) $AlCl_3$ (0.37 g, 2.78 mmol) was added portion wise to a suspension of $LiAlH_4$ (0.11 g, 2.90 mmol) in THF (10 mL) at 0 °C and this was stirred for 5 min followed by the drop wise addition of a solution of **4b** (675 mg, 1.44 mmol) in THF (5 mL). The reaction mixture was allowed to warm to room temperature, stirred overnight, cooled with an ice bath and quenched with a saturated solution of Na_2CO_3 in water until the foaming stopped. Subsequently, the suspension was filtered, the filtrate was dried over anhydrous $MgSO_4$, filtered again and evaporated in vacuo. Purification by flash chromatography (5% Et_3N in EtOAc) afforded 183 mg (39%) of the free base as an oil. This was converted to the fumaric salt as described for **7** to give 199 mg (24%) of **4c** as a light brown solid. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.54-1.75 (m, 2H), 1.82-1.93 (m, 2H), 2.05-2.18 (m, 4H), 2.27-2.39 (m, 2H), 2.59-2.68 (m, 2H), 2.86 (s, 2H),2.92-2.99 (m, 2H), 3.03-3.19 (m, 6H), 4.11 (s, 2H),6.60 (s, 2H), 6.73 (t, *J* = 7.3 Hz, 1H), 6.89-7.04 (m, 6H), 7.11-7.39 (m, 5H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 24.06, 31.36, 32.79, 33.75, 38.67, 48.42, 50.68, 54.91, 60.22, 119.50, 121.68, 125.47, 126.33, 126.68, 128.51, 128.59, 128.79, 129.20, 130.57, 131.95, 134.13, 135.82, 137.06, 141.06, 146.64, 149.77. Anal. RP-HPLC *Ib*: t_R = 4.43 min (purity 99%), *Ic* (total run time 20 min): t_R = 14.35 min (purity 100%), *IIe* (total runtime 30 min): t_R = 21.45 min (purity 96%). HRMS (EI) *m/z* calcd for $C_{30}H_{36}ClN_3$: 473.2598; found: 473.2579.

3-(4-Chlorophenyl)-1-(3-(11,12-dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)propyl)piperidin-3-ol fumarate (4d). Following method A using **11** (0.18 g, 0.63 mmol) and 3-(4-chlorophenyl)piperidin-3-ol **12** (0.11 g, 0.52 mmol), which was synthesized as described in the literature,[39] gave 77 mg of the free base as an oil. This was converted to the fumaric salt as described for **7** to give 50 mg (17%) of **4d** as a white solid. ^{1}H NMR (MeOH-d_4): δ 1.76-2.29 (m, 6H), 2.79-3.10 (m, 5H), 2.91 (s, 2H), 3.13-3.39 (m, 6H), 4.13 (s, 2H), 6.69 (s, 2H), 6.79-7.12 (m, 8H), 7.37-7.49 (m, 4H). ^{13}C NMR (MeOH-d_4): δ 20.44, 23.57, 34.56, 34.75, 35.80, 52.18, 53.72, 56.81, 61.36, 62.93, 71.47, 121.23, 124.28, 127.22, 127.77, 128.10, 128.49, 129.67, 129.94, 130.90, 132.24, 134.91, 136.16, 138.21, 139.02, 143.20,

144.30, 151.23, 171.08. Anal. RP-HPLC *Ib* (total run time 20 min): t_R = 6.78 min (purity 99%), *IIa* (total run time 30 min): t_R = 14.64 min (purity 98%). HRMS (EI) m/z calcd for $C_{29}H_{33}ClN_2O$: 460.2281; found: 460.2283.

5-(3-Chloropropyl)-5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine (11). 5,6,11,12-Tetrahydrodibenzo[*b*,*f*]azocine **10** (0.42 g, 2.01 mmol), which was synthesized as previously described in the literature,[38] and Na_2CO_3 (0.42 g, 3.96 mmol) were added in a 10 mL microwave vessel and 1-bromo-3-chloropropane (3 mL) was added. This solution was reacted in the microwave for 15 min at a temperature of 200 °C (settings: ramp time 5 min, hold time 15 min, power 200 watt, pressure 17.2 bar) and filtered. The solvent was evaporated in vacuo and the residue was purified by flash chromatography (15% DCM in hexane) to give 228 mg (40%) of **11** as a colourless oil. ^{1}H NMR ($CDCl_3$): δ 1.85-1.98 (m, 2H), 3.04-3.48 (m, 8H), 4.15 (s, 2H), 6.80-7.24 (m, 8H).

1-(3-(Benzyl(phenyl)amino)propyl)-4-(4-chlorophenyl)piperidin-4-ol hemifumarate (18a). Following method C starting with *N*-benzylaniline gave 331 mg of the free base as an oil. This was converted to the fumaric salt as described for **7** and recrystallized from MeOH/Et_2O to give 261 mg (24% over two steps) of **18a** as white crystals. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.53-1.72 (m, 2H), 1.74-1.97 (m, 2H), 2.00-2.21 (m, 2H), 2.41-2.70 (m, 4H), 2.82-3.01 (m, 2H), 3.26-3.48 (t, *J* = 7.4 Hz, 2H), 4.42 (s, 2H), 6.49-6.88 (m, 5H), 6.98-7.42 (m, 11H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 22.90, 36,45, 48.43, 54.32, 54.76, 69.23, 112.30, 116.32, 126.13, 126.43, 126.61, 128.02, 128.34, 129.04, 135.15, 138.49, 146.62, 148.14, 170.21. Anal. RP-HPLC *Ib*: t_R = 10.01 min (purity 100%), *IIb* (total run time 30 min): t_R = 8.05 min (purity 100%), *IIb* (total run time 30 min): t_R = 18.22 min (purity 100%). HRMS (EI) m/z calcd for $C_{27}H_{31}ClN_2O$: 434.2125; found: 434.2113.

1-(3-(Benzyl(4-chlorophenyl)amino)propyl)-4-(4-chlorophenyl)piperidin-4-ol hemifumarate (18b). Following method A starting with benzaldehyde and 4-chloroaniline gave 105 mg of the free base as an oil. This was converted to the fumaric salt as described for **7** and recrystallized from Et_2O/MeOH to give 112 mg of **18b** as white crystals (6% over 3 steps)
^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.61-2.05 (m, 4H), 2.13-2.37 (m, 2H), 2.53-2.86 (m, 2H), 2.99-3.12 (m, 2H), 3.37 (t, *J* = 7.1 Hz, 2H), 4.44 (s, 2H), 6.46-6.68 (m, 2H), 6.72 (s, 2H), 7.02-7.39 (m, 11H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 22.64, 36.16, 48.38, 48.70, 54.54, 69.37, 113.62, 121.22, 126.02, 126.40, 126.86, 128.26, 128.49, 128.88, 132.73, 135.35, 137.92, 145.84, 146.72, 170.93. Anal. RP-HPLC *Ib* : t_R = 5.03 min (purity 100%), *IIa* (total run time 30 min): t_R = 14.46 min (purity 98%). HRMS (EI) m/z calcd for $C_{27}H_{30}Cl_2N_2O$: 468.1735; found: 468.1718.

4-(4-Chlorophenyl)-1-(2-(8-(methylthio)-10,11-dihydro-dibenzo[*b*,*f*]thiepin-10-yloxy)-ethyl)piperidin-4-ol fumarate (24). (i) 4-(Methylthio)benzenethiol (5.24 g, 20.0 mmol) was added to a solution of 2-(2-iodophenyl)acetic acid **19** (3.12 g, 20.0 mmol) and KOH (4.49 g, 80.0 mmol) in

water (60 mL). After the addition of Cu (205 mg), the reaction mixture was refluxed for 48h, cooled to room temperature and filtered. The filtrate was acidified with 1.0 M HCl, filtered and the residue was dried under vacuum to yield 4.83 g (83%) of 2-(2-(4-(methylthio)phenylthio)phenyl)acetic acid **20** as a pink solid. ^{1}H NMR ($CDCl_3$): δ 2.43 (s, 3H), 7.12-7.35 (m, 8H).

(ii) PPA (50.0 g) was added to a solution of **20** (4.83 g, 16.6 mmol) in toluene (30 mL) and the reaction mixture was heated and refluxed for 18h. Water (50 mL) was added, the organic layer was separated and the water layer was extracted with EtOAc (3 x 30 mL). The combined organic extracts were washed with 5% NaOH (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 3.50 g (77%) of 8-(methylthio)dibenzo[*b*,*f*]thiepin-10(11*H*)-one **21** as a brown oil. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H), 4.36 (s, 2H), 7.13-7.64 (m, 6H), 8.02 (d, J = 2.3 Hz, 1H).

(iii) A solution of **21** (3.50 g, 12.8 mmol) and $NaBH_4$ (1.01 g, 26.7 mmol) in MeOH (100 mL) was stirred overnight at room temperature. The reaction mixture was evaporated in vacuo, water was added (50 mL) and the water layer was extracted with Et_2O (3 x 25 mL). The combined organic extracts were washed with water (3 x 50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 3.14 g (89%) of 8-(methylthio)-10,11-dihydrodibenzo[*b*,*f*]thiepin-10-ol **22** as a brown solid. ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H), 3.31-3.49 (m, 1H), 3.69-3.78 (m, 1H), 5.49 (br s, 1H), 6.95-7.49 (m, 7H).

(iv) $BF_3{\cdot}(Et)_2O$ (1.60 mL, 12.6 mmol) was added drop wise to a solution of **22** (3.14 g, 11.4 mmol) and 2-bromoethanol (1.22 mL, 17.2 mmol) in toluene (30 mL) and the reaction mixture was stirred for 1h. Water (25 mL) was added and the water layer was extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with water (25 mL), 5% NaOH (2 x 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 3.37 g of 11-(2-bromoethoxy)-2-(methylthio)-10,11-dihydrodibenzo[*b*,*f*]thiepine **23** as a brown oil. The crude product was used without further purification.

(v) Following method B using crude **23** (0.57 g, 1.49 mmol) and 4-(4-chlorophenyl)piperidin-4-ol **9** (0.21 g, 1.00 mmol) gave 88 mg of a light yellow oil. This was converted to the fumaric salt as described for **7** to give 101 mg (16%) of **24** as a white solid. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.67-1.80 (m, 2H), 2.21-2.57 (m, 5H), 2.78-3.31 (m, 6H), 3.42-3.62 (m, 2H), 3.78-3.99 (m, 2H), 5.22-5.33 (m, 1H), 6.73 (s, 2H), 7.00-7.41 (m, 12H). ^{13}C NMR ($CDCl_3$/DMSO-d_6): δ 15.53, 35.91, 38.84, 48.92, 49.19, 56.61, 64.57, 68.79, 77.22, 125.02, 125.61, 126.28, 127.43, 128.16, 129.64, 130.97, 131.39, 131.82, 132.58, 133.66, 134.94, 137.86, 138.80, 142.22, 146.10, 169.42. Anal. RP-HPLC *Ia*: t_R = 7.76 min (purity 99%), *IIa* (total run time 30 min): t_R = 19.48 min (purity 97%). HRMS (EI) m/z calcd for $C_{28}H_{30}ClNO_2S_2$: 511.1406; found: 511.1405.

2-(4-(3-(11,12-Dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)propyl)-piperazin-1-yl)ethanol dimaleate (25). This compound was obtained from our in-house database. ^{1}H NMR (MeOH-d_4): δ 1.79-1.85 (m, 2H), 2.72 (t, *J* = 7.7 Hz, 2H), 2.86-3.11 (m, 12H), 3.20-3.41 (m, 4H), 3.74 (t, *J* = 5.3 Hz, 2H), 4.19 (s, 2H), 6.27 (s, 4H), 6.86-6.90 (m, 1H), 6.98- 7.11 (m, 7H). ^{13}C NMR (MeOH-d_4): δ 24.93, 34.62, 35.62, 51.78, 52.07, 52.13, 56.21, 57.96, 59.73, 62.80, 121.62, 124.56, 127.16, 128.05, 128.56, 130.17, 130.17, 130.89, 132.32, 134.52, 138.50, 143.12, 170.06. Anal. RP-HPLC *Ic*: t_R = 4.56 min (purity 100%), *IId*: t_R = 4.00 min (purity 100%). HRMS (EI) m/z calcd for $C_{24}H_{33}N_3O$: 379.2624; found: 379.2619.

1-(11,12-Dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-3-(4-methylpiperazin-1-yl)propan-1-one dihydrochloride trihydrate (26). This compound was obtained from our in-house database. ^{1}H NMR (MeOH-d_4): δ 2.32-2.51 (m, 1H), 2.62-3.04 (m, 6H), 3.12-3.38 (m, 6H), 4.44-4.65 (m, 6H), 4.20 (d, *J* = 14.7 Hz, 1H), 5.68 (d, *J* = 14.7 Hz, 1H), 6.94-7.28 (m, 8H). ^{13}C NMR (MeOH-d_4): δ 30.40, 32.25, 35.64, 43.31, 50.15, 51.10, 53.83, 54.25, 127.35, 129.18, 129.20, 129.81, 130.28, 130.61, 131.20, 132.73, 135.50, 140.22, 141.05, 141.78, 170.58. Anal. RP-HPLC *Ic*: t_R = 4.11 min (purity 100%), *IId*: t_R = 3.85 min (purity 100%). HRMS (EI) m/z calcd for $C_{23}H_{29}N_3O$: 363.2311; found: 363.2307.

1-(11,12-Dihydrodibenzo[*b*,*f*]azocin-5(6*H*)-yl)-3-(4-(2-hydroxyethyl)piperazin-1-yl)-propan-1-one dimaleate (27). This compound was obtained from our in-house database. ^{1}H NMR (MeOH-d_4): δ 2.11-2.51 (m, 1H), 2.75-2.98 (m, 8H), 3.02-3.33 (m, 10H), 3.80 (t, *J* = 5.2 Hz, 2H), 4.17 (d, *J* = 14.7 Hz, 1H), 5.68 (d, *J* = 14.6 Hz, 1H), 6.28 (s, 4H), 6.99-7.19 (m, 8H).^{13}C NMR (MeOH-d_4): δ 32.18, 32.34, 35.61, 51.31, 52.49, 53.67, 54.17, 57.08, 59.53, 127.34, 128.98, 129.13, 129.66, 130.11, 130.58, 131.16, 132.57, 134.38, 135.67, 140.82, 140.96, 141.69, 170.00, 172.34. Anal. RP-HPLC *Ic*: t_R = 3.86 min (purity 100%), *IIe*: t_R = 6.00 min (purity 100%). HRMS (EI) m/z calcd for $C_{24}H_{31}N_3O_2$: 393.2416; found: 393.2408.

Pharmacology

[^{125}I]CCL5 binding experiments and [^{3}H]inositol phosphate accumulation assays were performed as previously described in the literature.[29]

References

1. Murphy, P. M. Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat. Immunol.* **2001**, *2*, 116-122.
2. Smit, M. J.; Vink, C.; Verzijl, D.; Casarosa, P.; Bruggeman, C. A.; Leurs, R. Virally encoded G protein-coupled receptors: targets for potentially innovative anti-viral drug development. *Curr. Drug Targets* **2003**, *4*, 431-441.
3. Davison, A. J.; Dargan, D. J.; Stow, N. D. Fundamental and accessory systems in herpesviruses. *Antiviral Res.* **2002**, *56*, 1-11.
4. Sodhi, A.; Montaner, S.; Gutkind, J. S. Viral hijacking of G-protein-coupled-receptor signalling networks. Nat. *Rev. Mol. Cell Biol.* **2004**, *5*, 998–1012.

5. Vischer, H. F.; Leurs, R.; Smit, M. J. HCMV-encoded G-protein-coupled receptors as constitutively active modulators of cellular signaling networks. *Trends Pharmacol. Sci.* **2006**, *27*, 56-63.
6. Chee, M. S.; Satchwell, S. C.; Preddie, E.; Weston, K. M.; Barrel, B. G. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature* **1990**, *344*, 774-777.
7. Britt, W. J.; Alford, C. A. In Fields Virology 3rd ed.; Fields, B. N.; Knipe, D. M.; Chanock, R. N., Ed.; Lippincott-Raven: Philadelphia, 1996; pp 2493-2523.
8. Jarvis, M. A.;Nelson, J. A. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Curr. Opin. Microbiol.* **2002**, *5*, 403-407.
9. Zhou, Y. F.; Leon, M. B.; Waclawiw, M. A.; Popma, J. J.; Yu, Z. X.; Finkel, T.; Epstein, S. E. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **1996**, *335*, 624-630.
10. Melnick, J. L.; Hu, C.; Burek, J.; Adam, E.; DeBakey, M. E. Cytomegalovirus DNA in arterial walls of patients with atherosclerosis. *J. Med. Virol.* **1994**, *42*, 170-174.
11. Valantine, H. A. The role of viruses in cardiac allograft vasculopathy. *Am. J. Transplant.* **2004**, *4*, 169-177.
12. Soderberg-Naucler, C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J. Intern. Med.* **2006**, *259*, 219-246.
13. Streblow, D. N.; Soderberg-Naucler, C.; Vieira, J.; Smith, P.; Wakabayashi, E.; Ruchti, F.; Mattison, K.; Altschuler, Y.; Nelson, J. A. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **1999**, *99*, 511-520.
14. Deayton, J. R.; Sabin, C. A.; Johnson, M. A.; Emery, V. C.; Wilson, P.; Griffiths, P. D. Importance of cytomegalovirus viraemia in risk of disease progression and death in HIV-infected patients receiving highly active antiretroviral therapy. *Lancet*, **2004**, *363*, 2116–2121.
15. Pleskoff, O.; Treboute, C.; Belot, A.; Heveker, N.; Seman, M.; Alizon, M. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **1997**, *276*, 1874-1878.
16. Pease, J. E.; Murphy, P. M. Microbial corruption of the chemokine system: an expanding paradigm. *Semin. Immunol.* **1998**, 10, 169-178.
17. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
18. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
19. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.
20. Kledal, T. N.; Rosenkilde, M. M.; Schwartz, T. W. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett.* **1998**, *441*, 209-214.
21. Billstrom, M. A.; Lehma, L. A.; Scott Worthen, G. Depletion of extracellulair RANTES during human cytomegalovirus infection of endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 163-167.
22. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
23. Casarosa, P; Bakker, R. A.; Verzijl, D.; Navis, M.; Timmerman, H.; Leurs, R.; Smit, M. J. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. *J. Biol. Chem.* **2001**, *276*, 1133-1137.
24. McLean, K. A.; Holst, P. J.; Martini, L.; Schwartz, T. W.; Rosenkilde, M. M. Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. *Virology* **2004**, *325*, 241-251.
25. Waldhoer, M.; Kledal, T. N.; Farell, H.; Schwartz, T. W. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. *J. Virol.* **2002**, *76*, 8161-8168.
26. Schall, T. J.; McMaster, B. E.; Dairaghi, D. J. Modulators of US28. World (PTC) Patent WO0217900, **2002**.
27. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
28. Hulshof, J.W.; de Esch, I. J. P.; Leurs, R. Synthesis and structure-Activity relationship of the first nonpeptidergic inverse agonists for the human cytomegalovirus encoded chemokine receptor US28. *J. Med. Chem.* **2005**, *48*, 6461-6471.

29. Hulshof J. W.; Vischer, H. F.; Verheij, M. H.; Fratantoni, S. A.; Smit, M. J.; de Esch, I. J. P.; Leurs, R. Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28. *Bioorg. Med. Chem.* **2006**, *14*, 7213-7230.
30. Lesniewski, M.; Das, S.; Skomorovska-Prokvolit, Y.; Wang, F.-Z.; Pellett, P. E. Primate cytomegalovirus US12 gene family: a distinct and diverse clade of seven-transmembrane proteins. *Virology* **2006**, *354*, 286-298.
31. Milligan, G.; Bond, R. A.; Lee, M. Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.* **1995**, *16*, 10-13.
32. Leurs, R.; Smit, M. J.; Alewijnse, A. E.; Timmerman, H. Agonist-independent regulation of constitutively active G-protein-coupled receptors. *Trends Biochem. Sci.* **1998**, *23*, 418-422.
33. Kenakin, T. P. The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol. Rev.* **1996**, *48*, 413-416.
34. Smit, M. J.; Vischer, H. F.; Bakker, R. A.; Jongejan, A.; Timmerman, H.; Pardo, L.; Leurs, R. Pharmacogenomic and structural analysis of constitutive G protein-coupled receptor activity. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 53-87.
35. Smit, M. J.; Leurs, R.; Alewijnse, A. E.; Blauw, J.; Amerongen, G. P. V.; Vandevrede, Y.; Roovers, E.; Timmerman, H. Inverse agonism of histamine H2 antagonist accounts for upregulation of spontaneously active histamine H2 receptors. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 6802-6807.
36. Kenakin, T. P. Efficacy at G-protein-coupled receptors. *Nat. Rev. Drug Discov.* **2002**, *1*, 103-110.
37. Buzas. A. Synthesis and psychoanaleptic properties of new compounds structurally related to diphenhydramine. *J. Med. Chem.* **1980**, *23*, 149-153.
38. Monro, A. M.; Quinton, R. M.; Wrigley, T. I. Some Analogs of Imipramine, *J. Med. Chem.* **1963**, *6*, 255-261.
39. Kumagai, T.; Okubo, T.; Kataoka-Okubo, H.; Chaki, S.; Okuyama, S.; Nakazato, A. Synthesis and structure-affinity relationships of 4-(5-aryl-1,2,3,6-tetrahydropyridino)pyrimidine derivatives as corticotropin-releasing factor(1) receptor antagonists. *Bioorg. Med. Chem.* **2001**, *9*, 1349-1355.
40. Kwartler, C. E.; Lucas, P. The preparation of substituted 4-aminomethylpiperidines and their straight chain analogs. *J. Am. Chem. Soc.* **1947**, *69*, 2582-2586.
41. Jílek, J.; Pomykáček, J.; Dlabač, A.; Bartošová, M.; Protiva, M. Neuroleptics of the 8-methylthio-10-piperazino-10,11-dihydrodibenzo[*b*,*f*]thiepin series: new compounds and new procedures. *Coll. Czech. Chem. Commun.* **1980**, *45*, 504-516.
42. Jílek, J. O.; Metyšova, J.; Pomykáček, J.; Protiva, M. 8-Alkylthio-10-piperazinodibenzo[*b.f*]-thiepins. *Coll. Czech. Chem. Commun.* **1974**, *39*, 3338-3351.
43. Bártl, V.; Jílek, J.; Metyšova, J.; Valchář, M.; Dlabač, A.; Wildt, S.; Protiva, M. Neuroleptic 2-chloro-11-(2-piperazinoethoxy)-10,11-dihydrodibenzo[*b.f*]thiepins: synthesis and pharmacology. *Coll. Czech. Chem. Commun.* **1984**, *49*, 1810-1815.
44. Govoni, M; Lim, H. D.; El-Atmioui, D.; Menge, W. M.; Timmerman, H.; Bakker, R. A.; Leurs, R.; De Esch, I. J. P. A chemical switch for the modulation of the functional activity of higher homologues of histamine on the human histamine H3 receptor: effect of various substitutions at the primary amino function. *J. Med. Chem.* **2006**, *49*, 2549-2555.
45. Yao, X.; Parnot, C.; Deupi, X.; Ratnala, V. R. P.; Swaminath, G.; Farrens, D.; Kobilka, B. Coupling ligand structure to specific conformational switches in the β_2-adrenoceptor. *Nat. Chem. Biol.* **2006**, *2*, 417-422.
46. Vilardaga, J.P.; Steinmeyer, R.; Harms, G. S.; Lohse, M. J. Molecular basis of inverse agonism in a G protein-coupled receptor. *Nat. Chem. Biol.* **2005**, *1*, 25-28.

Chapter 7

Novel 2-benzyl-2,3-dihydro-1*H*-inden-1-amines as inverse agonists for the chemokine receptor US28

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Abstract

Screening of our in-house compound collection afforded **1a** {2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-amine} as a novel inverse agonist acting on the human cytomegalovirus-encoded receptor US28. The structure of compound **1a** provided some interesting points for lead optimization. Firstly, the influence of the two chloro substituents was investigated by the introduction of different substitutions in the benzyl group of **1a**. Secondly, a series of analogues with various cyclic ring systems or small groups attached to the amine group were synthesized. Pharmacological characterization of these analogues revealed that 2,4-dichloro substitution in the benzyl group is preferred. All compounds with other substitution patterns in the benzyl ring were found to be less potent. Moreover, incorporation of the nitrogen atom of **1a** into different ring systems, namely a 4-(4-chlorophenyl)piperidin-4-ol moiety, a piperidine ring or a *N*-methylpiperazine group, is allowed in the structure.

Introduction

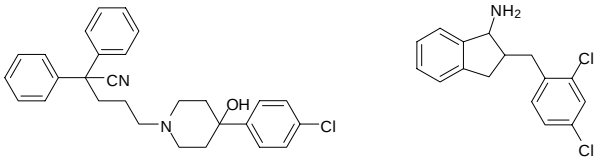
G protein-coupled receptors are a large family of cell-surface proteins that play important roles in the signal transduction in cells by the transmission of extracellular signals into intracellular responses. The GPCR family has emerged as one of the most important targets for therapeutic intervention, and currently many known marketed drugs are targeted on GPCRs.[1] Several herpesviruses have pirated cellular GPCR-encoding genes and modified their functional properties for their own benefit. Especially chemokine receptors have been pirated from their host to be incorporated in the viral genome to affect chemokine signaling to guarantee replication and survival of the virus. Only a couple of these viral GPCRs (vGPCRs) have so far been demonstrated to be functional chemokine receptors in respect of signal transduction and chemokine binding properties.[2] In contrast to their mammalian homologues, several of these vGPCRs activate various signaling pathways in a constitutively active manner. This ligand-independent signaling of several vGPCRs and the putative roles of chemokines and chemokine receptors in inflammatory diseases and tumour metastasis indicates that viral-encoded chemokine receptors may play an important role in virus-associated diseases.[3,4]

The genome of human cytomegalovirus (HCMV) encodes several GPCRs,[5-7] of which US28 is currently the most extensively characterized. Infection with HCMV has been associated with vascular diseases,[8] severe birth defects,[9] inflammatory and autoimmune diseases[10] as well as cancer.[11,12] The exact role of US28 during viral infection has not been elucidated yet, but the receptor has been shown to exhibit several interesting pharmacological features. The 30% amino acid sequence homology[13] of US28 with the human CCR1 chemokine receptor suggests that the virus exploits chemokine signal transduction pathways to intervene in the host immune system through chemokine mimicry.[14] US28 can bind a number of inflammatory CC chemokines[13,15,16] and eliminates them efficiently from the extracellular environment via constitutive receptor internalization, thereby escaping immune surveillance.[17,18] Moreover, the high affinity binding of the membrane-bound CX3C chemokine CX3CL1 is suggested to play a role in the cell to cell transfer of HCMV.[19] Another potential way of spreading the virus through the human body is via the migration of vascular

smooth muscle cells by US28 upon binding of the CC chemokines CCL2 and CCL5.[20] The migration of smooth muscle cells plays a significant role in the formation of atherosclerotic and restenotic lesions, thereby providing a possible link between HCMV and the development of vascular diseases. Additionally, US28 is suggested to play a role in the dissemination of the HIV-1 virus through the body by its in vitro co-receptor activity.[21] Recently, it was discovered that US28 might act as a viral oncogene by causing transformations and promoting tumorigenesis in mice.[22] The constitutive signaling of US28 was shown to be of importance in the early beginning of tumor formation. Mice that were injected with NIH-3T3 cells stably transfected with the US28-R129A mutant receptor, which lacks constitutive signaling, showed a delayed and attenuated tumor formation, further emphasizing the physiological relevance of the ligand-independent signaling after viral infection.

In summary, US28 has been shown to display many interesting features in vitro, thereby providing a causative link between US28 and several diseases resulting from infection with HCM. However, the lack of in vivo models hampers the analysis of the function of US28 in physiological relevant systems so far.

To further investigate the relevance of the constitutive signaling of US28 during viral infection we were aiming to identify inverse agonists that are able to inhibit the ligand-independent signaling of the receptor. These molecules are valuable tools in elucidating the role of US28 in the aforementioned pathologies related to HCMV infection. Previously, VUF2274 was identified as the very first inverse agonist acting on the viral-encoded receptor US28 with IC_{50} and EC_{50} values of 4.9 and 3.2 μM (Figure 1).[23]



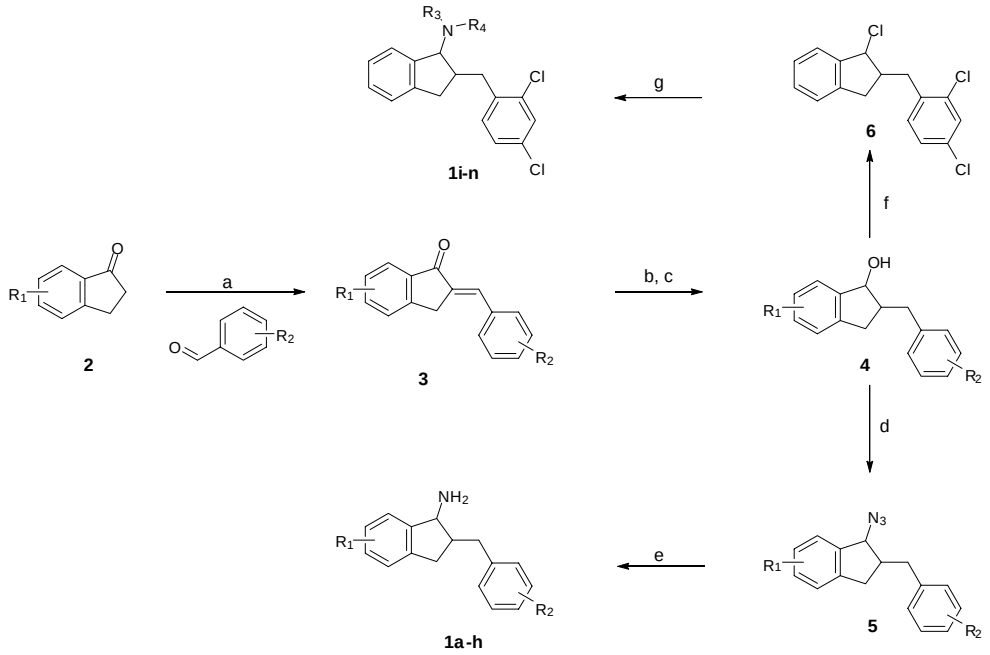
VUF2274 **1a**

Figure 2. Chemical structures of VUF2274 and hit compound **1a**.

Interestingly, this molecule was able to inhibit the US28-mediated HIV entry in cells when cotransfected with CD4. Subsequent lead optimization of VUF2274 resulted in the disclosure of the very first structure-activity relationships for inverse agonism on US28.[24,25] Novel chemotypes could provide new opportunities for the development of potent and selective inverse agonists, eventually leading to promising therapeutics for clinical anti-viral intervention. Thus, a selection of our in-house compound collection was screened (Chapter 5) and this resulted in the identification of Z193 (**1a**) as a novel inverse agonists for the HCMV-encoded receptor US28 (Figure 1). In this study, a series of analogues of compound **1a** was synthesized to explore the first structure-activity relationships for the interaction of these promising novel inverse agonists targeting the HCMV-encoded receptor US28.

Chemistry

A general synthetic route for the preparation of compounds **1a**-**1n** is outlined in Scheme 1. The substituted 2-(phenylmethylene)-2,3-dihydro-1*H*-inden-1-ones **3** were synthesized in high yields by treating indanones **2** with the appropriate substituted benzaldehydes in a saturated solution of K_2CO_3 in EtOH.[26] Reduction of both the carbonyl group as well as the double bond of intermediates **3** in one pot with lithium aluminium hydride was not successful. Therefore, hydrogenation of the double bond and subsequent reduction of the carbonyl group with sodium borohydride afforded the substituted 2-benzyl-2,3-dihydro-1*H*-inden-1-ols **4** as 1:1 mixture of both diastereomers in moderate to high yields. Intermediates **4** were converted to the corresponding 1-azido-2-benzyl-2,3-dihydro-1*H*-indenes **5** in the presence of diphenyl phosphorazidate (DPPA) and 2,3,4,6,7,8,9,10-octahydropyrimidol[1,2-*a*]azepine (DBU) in THF.[27,28] For some of the azide intermediates it was possible to separate the two diasteromers via column purification. Hydrogenation of the azide group with palladium on activated carbon afforded target compounds **1a**-**1h** (Method A). The separation of the two diastereomers could not be accomplished after the last step of the synthesis route.



Scheme 1. Synthetic pathway for the synthesis of **1a**-**1n**. Reagents and conditions: (a) K_2CO_3, EtOH; (b) 10% Pd/C, H_2, MeOH; (c) $NaBH_4$, MeOH; (d) DPPA, DBU, THF; (e) 10% Pd/C, H_2, MeOH; (f) $SOCl_2$, DCM; (g) Amine, NaI, CH_3CN, 90°C.

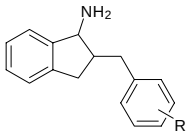
Treatment of 2-benzyl-2,3-dihydro-1*H*-inden-1-ol derivatives **4** with thionyl chloride gave a 1:1 mixture of the two diastereomers of 1-chloro-2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-indenes **6** in a quantitative yield. Alkylation of chloro derivatives **6** with the appropriate amine groups in the presence of NaI, Na_2CO_3, and CH_3CN at reflux temperature or in the microwave, as described in Chapter 3, gave mainly elimination of HCl.[25] However, *N*-alkylation of the different commercially available amines with chloro intermediates **6** was successful by heating the corresponding amine in CH_3CN to reflux temperature in the presence of Na_2CO_3 and NaI and subsequent addition of the appropriate chloride **6** when reflux temperature was reached. This resulted in the synthesis of target compounds **1i**-**n** in moderate to low yields (Method B). This synthesis route resulted in the formation of only one diastereomer of the desired compounds. Most likely, the nucleophiles, e.g. *N*-methylpiperazine, are sterically less hindered if they approach the 5-membered ring of intermediates **6** opposite the 2,4-dichlorobenzyl group, as in (1*R*,2*S*)-**6** and (1*S*,2*R*)-**6**. This S_N2

substitution reaction gives inversion of configuration, resulting in the formation of (1*R*,2*R*)-**1m** and (1*S*,2*S*)-**1m**. Approach of the amine from the other side, as in (1*R*,2*R*)-**6** and (1*S*,2*S*)-**6**, is less favourable, giving presumably elimination of HCl instead of reaction to the desired product. Unfortunately, additional 2D NMR studies to characterize the different diastereomers were not successful and could not be used to prove the hypothesis.

Results and discussion

To identify novel inverse agonist for US28, a selection of compounds from our in-house database was screened for their ability to modulate the basal signaling of the receptor. This resulted in the identification of compound **1a** as a novel nonpeptidergic inverse agonist acting on US28. Starting from this, a series of analogues of compound **1a** were synthesized, which were evaluated for their potential to dose-dependently displace [^{125}I]CCL5 binding to US28. The inverse agonistic properties of a selection of compounds were investigated by testing their potential to inhibit the US28-mediated constitutive inositol phosphate production in SVEC4-10 cells.

Table 1. Chemical structures and pharmacological properties of compounds **1a**-**1h** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	Code or VUF number	R	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1a	Z193	2,4-Cl_2	9.5 (6.8 – 12.3)	11.1 (8.3 – 13.8)
1b	VUF10482	2,4-Me_2	17.7 (14.5 – 20.9)	13.7 (9.1 – 18.2)
1c	VUF10481	2,4-$(OMe)_2$	> 100	> 100
1d	VUF10486	2-OMe	37.3 (28.8 – 45.7)	42.7 (41.7 – 43.7)
1e	VUF10478	4-OMe	> 100	> 100
1f	VUF10476	3-Me-4-OMe	59.7 (50.1 – 69.2)	16.4 (14.1 – 18.6)
1g	VUF10480	4-NH(C=O)CH_3	> 100	> 100
1h	VUF10477	H	80.6 (32.4 – 128.8)	> 100

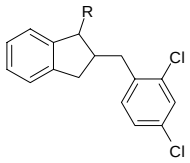
[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

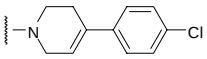
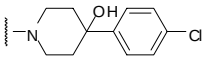
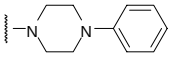
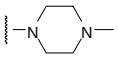
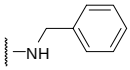
The influence of the substitution pattern of the benzyl group was investigated in compounds **1b**-**1h** (Table 1). Interestingly, all the compounds with variations in the benzyl group, including the unsubstituted analogue **1h**, are found to be less potent. Replacements of the electron donating chloro substituents of **1a** by electron releasing methyl groups (**1b**) results in a small decrease in binding affinity. However, introduction of the two methoxy groups in compound **1c** is not allowed to maintain affinity or efficacy on US28. To investigate the influence of the position of the methoxy groups compounds **1d** and **1e** were synthesized. Introduction of a methoxy group at the ortho position of the phenyl ring (**1d**) only causes a 3-fold drop in binding affinity and efficacy compared to lead compound **1a**, while a methoxy group in the para position results in a complete loss of activity for the receptor. Interestingly, introduction of an additional methyl group at the meta position of the phenyl ring results in a partial recovery of the binding affinity, while the efficacy is comparable to lead compound **1a**.

The influence of an acetamide group at the para position of the phenyl ring was investigated by the synthesis of analogue **1f**. As can be seen in Table 1, it seems that this substituent is not permitted at this position. Removal of all substituents in the phenyl ring (**1h**) is not beneficial for activity either, as this compound has a more than 7-fold lower binding affinity than lead compound **1a**. Taken together, several analogues were synthesized to optimize the substitution pattern in the benzyl group of lead compound **1a**, but all variations in this part of the molecule did not improve the binding affinity or activity on US28.

To study the importance of the amine group of hit compound **1a**, different cyclic ring systems or small groups were introduced (Table 2). Introduction of the unsaturated tetrahydropyridine moiety in **1i** results in a complete loss of affinity and activity, while incorporation of the nitrogen atom into other ring systems, e.g. in a 4-(4-chlorophenyl)piperidin-4-ol moiety (**1j**) or a piperidine ring (**1k**) leads to a binding affinity comparable to compound **1a**. Previously, the same effect in binding affinity between 4-(4-chlorophenyl)piperidin-4-ol substitution and a tetrahydropyridine moiety was observed in VUF2274 analogues.[24] Interestingly, small improvements in efficacy are observed for both **1j** and **1k** compared to lead compound **1a**.

Table 2. Chemical structures and pharmacological properties of compounds **1a** and **1i**-**1n** for the HCMV-encoded receptor US28. The values are represented as the mean and the interval of the IC_{50} and EC_{50} values of at least three independent experiments.



no.	Code or VUF number	R_1	IC_{50} (μM)[a]	EC_{50} (μM)[b]
1a	Z193		9.5 (6.8 – 12.3)	11.1 (8.3 – 13.8)
1i	10385		> 100	> 100
1j	10384		11.0 (10.0 – 12.0)	6.0 (4.0 – 7.9)
1k	10356		13.4 (11.2 – 15.5)	2.6 (2.1 – 3.0)
1l	10357		> 100	> 100
1m	10386		8.1 (6.6 – 9.5)	12.1 (9.1 – 15.1)
1n	10419		> 100	> 100

[a] [^{125}I]CCL5 displacement. [b] Inhibition of [^{3}H]inositol phosphate production.

Introduction of the *N*-phenylpiperazine moiety in analogue **1l** is detrimental for the binding affinity and efficacy, while the smaller *N*-methylpiperazine group in **1m** results in a compound with an activity comparable to compound **1a**. Attachment of a benzyl group to the nitrogen atom as in **1n** is not allowed to maintain binding affinity or efficacy on the receptor.

In summary, incorporation of the nitrogen atom of **1a** into several ring systems, such as a 4-(4-chlorophenyl)piperidin-4-ol group, a piperidine ring or a *N*-methylpiperazine moiety, is permitted in the structure. In contrast, the introduction of the tetrahydropyridine moiety in **1i**, the *N*-phenylpiperazine group in **1l** and the benzyl substitution in **1n** are not allowed and cause a complete drop in affinity anf efficacy.

Conclusions

Screening of our in-house compound collection resulted in the identification of Z193 (**1a**) as a novel scaffold for inverse agonism on the viral-encoded receptor US28. To explore the first structure-activity relationships of these novel 2-benzyl-2,3-dihydro-1*H*-inden-1-amines different analogues were synthesized and pharmacologically evaluated. This hit optimization of **1a** revealed that 2,4-dichloro substitution in the benzyl group is prefered, as all compounds with other variations in the substitution pattern were found to be less potent. Moreover, incorporation of the nitrogen atom of **1a** into a 4-(4-chlorophenyl)piperidin-4-ol moiety, a piperidine ring or a *N*-methylpiperazine group, is allowed to maintain affinity and efficacy. These ligands possess an interesting new scaffold for inverse agonism on US28 and may provide useful information about ligand-receptor interactions that are important for binding and efficacy on the receptor.

Experimental section

General procedures. THF and DCM were freshly distilled from lithium aluminium hydride. All reactions were performed under an atmosphere of dry nitrogen. Microwave reactions were performed in a CEM Explorer single mode MW reactor equipped with auto sampler. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. J.T. Baker silica gel was used for flash chromatography. HRMS mass spectra were recorded on a Finnigan MAT 900 mass spectrometer by electron ionization (EI) mass spectrometry or on a Shimadzu IT TOF (ion trap – time-of flight) mass spectrometer by electron spray ionization (ESI) mass spectrometry. Melting points were measured on a MPA100 OptiMelt automated melting point system apparatus and were uncorrected. Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV-VIS detector set at 254 nm, with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph mass spectrometer. The analyses were performed using the following conditions; condition I: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 60% MeOH - 40% H_2O-0.1% formic acid (Method Ia); 50% MeOH - 50% H_2O-0.1% formic acid (Method Ib), 40% MeOH - 50% H_2O-0.1% formic acid (Method Ic) or 30% MeOH - 70% H_2O-0.1% formic acid (Method Id). Flow rate = 1.0 mL/min. Total run time 15 min unless otherwise stated. Condition II: a Xbridge(C18)5um column (100 mm x 4.9 mm) with 50% CH_3CN - 50% H_2O-0.1% formic acid (Method IIa); 40% CH_3CN - 60% H_2O-0.1% formic acid (Method IIb) or 30% CH_3CN - 70% H_2O-0.1% formic acid (Method IIc). Flow rate = 1.0 mL/min. Total run time 20 min. Condition III: a Xbridge(C18)5um

column (100 mm x 4.9 mm) with 60% CH_3CN - 40% H_2O with 10 % NH_4HCO_3/NH_4OH buffer pH 8 (Method IIIa) or 50% CH_3CN - 50% H_2O-0.1% formic acid (Method IIIb). Flow rate = 1.0 mL/min. Total run time 20 min. Compounds that were isolated as fumaric acid salts all showed an extra peak around two minutes. Fumaric acid blancs were used to determine the *t*R of fumaric acid. Purities calculated are based on RP HPLC-UV peak surface area of the compounds (disregarding the fumaric acid peak). Compound **1a** was taken from our in-house database.

General method A for the synthesis of target compounds 1a-1h. 2-(2,4-Dimethylbenzyl)-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1b). (i) 2,4-Dimethylbenzaldehyde (1.67 g, 10.0 mmol) and 1-indanone (1.33 g, 10.0 mmol) were added to a saturated solution of anhydrous K_2CO_3 in EtOH (25 mL).The resulting reaction mixture was stirred vigorously for 24 h, 37% HCl (2 mL) was added and the solvent was evaporated in vacuo. Water (50 mL) was added and the water layer was extracted with DCM (3 x 25 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo. The residue was recrystallized from EtOH to give 1.95 g (79%) of 2-(2,4-dimethylbenzylidene)-2,3-dihydro-1*H*-inden-1-one as white crystals. ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H), 2.43 (s, 3H), 3.95 (s, 2H), 6.98-7.11 (m, 2H), 7.31-7.60 (m, 3H), 7.80-7.95 (m, 2H).

(ii) A suspension of 2-(2,4-dimethylbenzylidene)-2,3-dihydro-1*H*-inden-1-one (1.14 g, 5.49 mmol) and 10% Pd/C (5 mg) in MeOH (10 mL) was hydrogenated with H_2 (atmospheric pressure) for 18 h. The reaction mixture was filtrated over Hyflo and the residue was washed with EtOH. The filtrate was evaporated in vacuo to give 2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-inden-1-one in a quantitative yield. ^{1}H NMR ($CDCl_3$): δ 2.28 (s, 3H), 2.30 (s, 3H), 2.41-2.59 (m, 1H), 2,72-3.05 (m, 2H), 3.10-3.22 (m, 1H), 3.37 (dd, *J* = 10.4 Hz and 3.9 Hz, 1H), 6.81-7.01 (m, 3H), 7.25-7.41 (m, 2H), 7.54 (d, *J* = 7.5 Hz, 1H), 7.77 (d, *J* = 7.6 Hz, 1H).

(iii) A solution of 2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-inden-1-one (1.15 g, 4.59 mmol) and $NaBH_4$ (0.17 g, 4.59 mmol) in MeOH (10 mL) was stirred overnight at room temperature. The reaction mixture was evaporated in vacuo, water was added (25 mL) and the water layer was extracted with EtOAc (3 x 20 mL). The combined organic extracts were washed with water (3 x 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give a quantitative yield of 2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-inden-1-ol as a 1:1 mixture of two diastereomers. ^{1}H NMR ($CDCl_3$): δ 1.70 (br s, 1H), 2.30 (s, 6H), 2.35-2.88 (m, 3H), 2.98-3.12 (m, 2H), 4.83-5.01 (m, 1H), 6.89-7.01 (m, 2H), 7.04-7.41 (m, 6H).

(iv) A solution of 2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-inden-1-ol (1.15 g, 4.59 mmol) and DPPA (1.39 mL, 6.41 mmol) in THF (10 mL) was stirred for 10 min, cooled to 0 °C and DBU (0.98 mL, 6.42 mmol) was added in a drop wise manner. The reaction mixture was allowed to warm to room

temperature and stirred for 18 h. The solvent was evaporated in vacuo, water (25 mL) was added and the water layer was extracted with Et_2O (3 x 15 mL). The combined organic extracts were washed with brine (25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo. Purification by column chromatography (hexane) afforded 763 mg (60%) of 1-azido-2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-indene as a 1:1 mixture of two diastereomers. ^{1}H NMR ($CDCl_3$): δ 2.29 (s, 3H), 2.30 (s, 3H), 2.51-2.84 (m, 4H), 2.90-3.11 (m, 1H), 4.52 and 4.70 (d, *J* = 4.7 and 5.0 Hz, 1H), 6.87-7.03 (m, 3H), 7.11-7.39 (m, 4H).

(v) A solution of 1-azido-2-(2,4-dimethylbenzyl)-2,3-dihydro-1*H*-indene (0.76 g, 2.74 mmol) and 10% Pd/C (3 mg) in MeOH (10 mL) was hydrogenated with H_2 (atmospheric pressure) for 3 days. The reaction mixture was filtrated over Hyflo, the residue was washed with EtOH and the filtrate was evaporated in vacuo. Purification by flash chromatography (2% Et_3N in hexane) gave 646 mg of the free base. This was dissolved in a solution of HCl gas in EtOH and the solvent was evaporated in vacuo. The hydrochloride salt was isolated by filtration and recrystallized from Et_2O/MeOH to give 665 mg (84%) of **1b** as a 1:1 mixture of two diastereomers. ^{1}H NMR (MeOH-d_4): δ 2.30 (s, 3H), 2.33 (s, 3H), 2.61-3.18 (m, 5H), 4.51 and 4.83 (d, *J* = 3.5 and 6.0 Hz, 1H), 6.92-7.15 (m, 3H), 7.21-7.40 (m, 3H), 7.49-7.63 (m, 1H). ^{13}C NMR (MeOH-d_4): δ 18.64, 20.02, 31.51, 35.25, 35.87, 43.08, 58.19, 125.44, 125.73, 126.78, 127.33, 129.29, 129.96, 131.25, 134.43, 136.07, 136.21, 138.73, 143.83 (one diastereomer). ^{13}C NMR (MeOH-d_4): δ 17.38, 18.59, 31.51, 35.25, 35.87, 45.21, 60.52, 125.18, 125.31, 127.48, 129.66, 131.27, 134.49, 136.27, 136.30, 137.94, 143.58 (other diastereomer). Anal. RP-HPLC *Ia* (total run time 30 min): t_R = 12.89 min (purity 100%), *IIb*: t_R = 11.79, 12.79 min (purity 100%). HRMS (EI) m/z calcd for $C_{18}H_{21}N$: 251.1674; found: 251.1665.

2-(2,4-Dimethoxybenzyl)-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1c). Yield: 539 g (17% yield over 5 steps) of **1c** as a 1:1 mixture of two diastereomers. ^{1}H NMR (MeOH-d_4): δ 2.58-3.14 (m, 5H), 3.78 and 3.79 (s, 3H), 3.81 and 3.82 (s, 3H), 4.43 and 4.69 (d, *J* = 4.1 and 5.8 Hz, 1H), 6.45-6.57 (m, 2H), 7.02-7.52 (m, 5H). ^{13}C NMR (MeOH-d_4): δ 35.44, 35.79, 45.35, 54.82, 54.84, 54.91, 57.98, 98.55, 104.89, 119.62, 125.36, 125.41, 127.24, 129.95, 131.31, 138.83, 144.09, 158.89, 160.45 (one diastereomer). ^{13}C NMR (MeOH-d_4): δ 28.70, 32.75, 43.45, 54.82, 54.84, 54.91, 60.26, 98.50, 104.61, 119.77, 124.92, 125.59, 127.32, 129.80, 130.80, 138.12, 143.62, 158.54, 160.52 (other diastereomer). Anal. RP-HPLC *Ia* : t_R = 8.47 and 9.81 min (purity 100%), *IIIb*: t_R = 5.29 and 5.61 min (purity 100%). HRMS (EI) m/z calcd for $C_{18}H_{21}NO_2$: 283.1572; found: 283.1582.

2-(2-Methoxybenzyl)-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1d). Yield: 439 mg (17% yield over 5 steps) of one diastereomer of **1d** as a white solid. ^{1}H NMR (MeOH-d_4): δ 2.52-2.80 (m, 2H), 2.85-3.15 (m, 3H), 3.85 (s, 3H), 4.71 (d, *J* = 5.9 Hz, 1H), 6.89-6.97 (m, 2H), 7.01-7.36 (m, 5H), 7.50 (d, *J* = 6.6 Hz, 1H). ^{13}C NMR (MeOH-d_4): δ 30.37, 36.41, 44.21, 55.82, 59.00, 111.76,

121.82, 126.30, 126.43, 128.28, 128.54, 129.17, 131.01, 131.43, 139.80, 145.96, 158.71. RP-HPLC *Ib*: t_R = 4.19 min (purity 100%), *IIb*: t_R = 3.95 min (purity 100%). HRMS (EI) m/z calcd for $C_{17}H_{19}NO$: 253.1467; found: 253.1476.

2-(4-Methoxybenzyl)-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1e). Yield: 439 mg (19% yield over 5 steps) of **1e** as a 1:1 mixture of two diastereomers. ^{1}H NMR (MeOH-d_4): δ 2.51-3.29 (m, 5H), 3.77 (s, 3H), 3.79 (s, 3H), 4.49 and 4.77 (d, *J* = 3.9 and 6.2 Hz, 1H), 6.80-6.94 (m, 2H), 7.08-7.52 (m, 6H). ^{13}C NMR (MeOH-d_4): δ 35.89, 38.28, 46.58, 54.73, 60.39, 114.14, 125.03, 125.67, 127.44, 129.75, 130.06, 131.55, 137.93, 143.47, 158.92 (one diastereomer). ^{13}C NMR (MeOH-d_4): δ 34.12, 35.33, 44.47, 54.73, 58.08, 114.11, 125.35, 125.46, 127.34, 129.92, 129.98, 131.49, 138.73, 143.82, 158.92 (other diastereomer). RP-HPLC *Ib*: t_R = 5.73 min (purity 100%), *IIc*: t_R = 5.48 min (purity 96%). HRMS (EI) m/z calcd for $C_{17}H_{19}NO$: 253.1467; found: 253.1459.

2-(4-Methoxy-3-methylbenzyl)-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1f). Yield: 470 mg (20% yield over 5 steps) of one diastereomer of **1g** as a white solid. ^{1}H NMR (MeOH-d_4): δ 2.42 (s, 3H), 2.42-3.09 (m, 5H), 3.80 (s, 3H), 4.77 (d, *J* = 6.0 Hz, 1H) 6.82-7.54 (m, 7H). ^{13}C NMR (MeOH-d_4): δ 15.35, 34.14, 35.37, 44.48, 54.84, 58.09, 110.21, 125.30, 125.46, 126.72, 131.04, 127.05, 127.31, 129.96, 130.89, 138.78, 143.86, 156.94. Anal. RP-HPLC *Ia*: t_R = 4.56 min (purity 100%), *IIa*: t_R = 4.33 min (purity 99%). HRMS (EI) m/z calcd for $C_{18}H_{21}NO$: 267.1623; found: 267.1623.

2-(4-Methoxy-3-methylbenzyl)-N-(4-((1-amino-2,3-dihydro-1*H*-inden-2-yl)methyl)-phenyl)acetamide (1g). Yield: 581 mg (23% yield over 5 steps) of **1f** as a 1:5 mixture of two diastereomers. (MeOH-d_4): δ 2.10 (s, 3H), 2.20-2.34 (m, 1H), 2.41-77 (m, 2H), 2.81-2.92 (m, 1H), 3.00-3.15 (m, 1H), 3.98 and 4.28 (d, *J* = 7.6 and 7.8 Hz, 1H), 6.98-7.51 (m, 8H). ^{13}C NMR (MeOH-d_4): δ 22.73, 36.38, 38.92, 52.76, 61.76, 120.45, 123.50, 124.48, 126.56, 127.41, 129.28, 136.94, 137.22, 141.91, 146.11, 170.55 (major diastereomer). ^{13}C NMR (MeOH-d_4): δ 22.73, 34.10, 35.19, 46.63, 58.46, 120.32, 124.23, 124.65, 124.77, 127.63, 129.17, 136.82, 137.34, 141.91, 146.11, 170.55 (minor diastereomer). RP-HPLC *Id* (total run time 30 min): t_R = 10.42 min (minor diastereomer), 13.62 min (major diastereomer) (purity 100%), *IId*: t_R = 5.21 min (minor diastereomer), 6.22 min (major diastereomer) (purity 100%). HRMS (EI) m/z calcd for $C_{18}H_{20}N_2O$: 280.1576; found: 280.1571.

2-Benzyl-2,3-dihydro-1*H*-inden-1-amine hydrochloride (1h). Yield: 350 mg (14% yield over 5 steps) of one diastereomer of **1h** as a white solid. ^{1}H NMR (MeOH-d_4): δ 2.52-2.66 (m, 1H), 2.70-3.05 (m, 3H), 3.09-3.20 (m, 1H), 4.79 (d, *J* = 6.4 Hz, 1H), 7.25-7.55 (m, 9H). ^{13}C NMR (MeOH-d_4): δ 35.03, 35.29, 44.29, 58.13, 125.29, 125.47, 125.61, 127.37, 128.73, 128.80, 130.02, 138.68, 139.59, 143.77. RP-HPLC *Ib*: t_R = 5.52 min (purity 100%), *IIc*: t_R = 5.94 min (purity 97%), *IIIa*: t_R = 3.37 min (purity 99%). HRMS (EI) m/z calcd for $C_{16}H_{17}N$: 223.1361; found: 223.1363.

General method B for the synthesis of target compounds 1i-1n. 1-(2-(2,4-Dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-yl)piperidine (1k) (i) 2,4-Dichlorobenzaldehyde (17.5 g, 100 mmol) and 1-indanone (13.2 g, 100 mmol) were added to a saturated solution of anhydrous K_2CO_3 in EtOH (250 mL). The resulting reaction mixture was stirred vigorously for 18 h and 37% HCl (20 mL) was added. The precipitate was isolated by filtration, washed with water and dried under vacuum to give 25.8 g (89%) of 2-(2,4-dichlorobenzylidene)-2,3-dihydro-1*H*-inden-1-one as white crystals. ^{1}H NMR ($CDCl_3$): δ 3.92 (s, 2H), 7.12-7.66 (m, 5H), 7.80-7.99 (m, 2H).

(ii) A suspension of 2-(2,4-dichlorobenzylidene)-2,3-dihydro-1*H*-inden-1-one (11.6 g, 40.1 mmol) and 10% Pd/C (40 mg) in MeOH (100 mL) was hydrogenated with H_2 (atmospheric pressure) for 6 h at a temperature of 50 °C. The reaction mixture was filtrated over Hyflo and the filtrate was evaporated in vacuo. Recrystallization from EtOH afforded 4.6 g (39%) of 2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-one as white crystals. ^{1}H NMR ($CDCl_3$): δ 2.65-2.88 (m, 2H), 2.95-3.20 (m, 2H), 3.27-3.40 (m, 1H), 7.06-7.20 (m, 2H), 7.26-7.40 (m, 3H), 7.56 (t, *J* = 7.4 Hz, 1H), 7.75 (d, *J* = 7.6 Hz, 1H).

(iii) A solution of 2-(2,4-dichorolbenzyl)-2,3-dihydro-1*H*-inden-1-one (1.86 g, 6.40 mmol) and $NaBH_4$ (0.17 g, 4.59 mmol) in MeOH (40 mL) was stirred for 3 h at room temperature. The reaction mixture was evaporated in vacuo, water was added (20 mL) and the water layer was extracted with EtOAc (3 x 15 mL). The combined organic extracts were washed with water (3 x 25 mL) and brine (25 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 1.84 (98%) of 2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-ol as a 1:1 mixture of two diastereomers. ^{1}H NMR ($CDCl_3$): δ 1.72 (br s, 1H), 2.41-3.02 (m, 5H), 4.92 (br s, 1H), 7.16-7.38 (m, 7H).

(iv) Thionyl chloride (0.75 mL, 10.3 mmol) was added to a solution of 2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-ol (1.84 g, 6.28 mmol) in toluene (30 mL) at 0 °C. The reaction mixture was stirred at room temperature for 30 min, heated to 55 °C and stirred for 1 h. The solution was cooled to room temperature, washed with ice-water (2 x 15 mL), dried over anhydrous Na_2SO_4, filtered and evaporated in vacuo to give 1.88 g (96%) of 1-chloro-2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-indene as a 1:1 mixture of two diastereomers. ^{1}H NMR ($CDCl_3$): δ 2.55-3.29 (m, 5H), 5.10 and 5.27 (d, *J* = 5.3 and 4.8 Hz, 1H), 7.08-7.41 (m, 7H).

(v) A solution of piperidine (70 μL, 0.71 mmol), NaI (75 mg, 0.50 mmol), and Na_2CO_3 (106 mg, 1.0 mmol) in CH_3CN (10 mL) was heated to reflux temperature and 1-chloro-2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-indene (0.16 g, 0.51 mmol) in CH_3CN (1 mL) was added. The reaction mixture was refluxed overnight, the solvent was removed in vacuo and the residue was diluted with water (15 mL) followed by an extraction with DCM (3 x 10 mL). The combined organic layers were dried over anhydrous $NaSO_4$, filtered, and evaporated in vacuo. Purification by flash chromatography (25% EtOAc in DCM) and recrystallization from EtOAc gave 90 mg (49%) of **1k** as white crystals. ^{1}H NMR ($CDCl_3$): δ 1.29-1.51

(m, 6H), 2.29-2.70 (m, 6H), 2.76-2.99 (m, 3H), 3.95 (d, *J* = 3.4 Hz, 1H), 7.03-7.22 (m, 5H), 7.29-7.41 (m, 3H). ^{13}C ($CDCl_3$): δ 24.61, 26.41, 37.00, 38.18, 38.80, 50.19, 76.04, 124.50, 125.88, 126.09, 126.65, 127.38, 129.12, 131.78, 132.15, 134.78, 137.23, 142.58. Anal. RP-HPLC *Ib* (total run time 30 min): t_R = 19.88 min (purity 100%), *IIb* (total run time 30 min): t_R = 13.77 min (purity 100%). HRMS (EI) m/z calcd for $C_{21}H_{23}Cl_2N$: 359.1208; found: 359.1199.

4-(4-Chlorophenyl)-1-(2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-yl)-1,2,3,6-tetrahydropyridine hydrochloride (1i). Yield: 50 mg (19%) of one diastereomer of **1i** as a light brown solid. ^{1}H NMR (MeOH-d_4): δ 2.61-2.92 (m, 6H), 3.15-3.39 (m, 3H), 3.51-3.81 (m, 2H), 4.61-4.69 (m, 1H), 6.01-6.07 (m, 1H), 7.31-7.54 (m, 10H), 7.67 (d, *J* = 7.1 Hz, 1H).^{13}C (MeOH-d_4): δ 26.22, 37.74, 38.29, 40.69, 47.72, 47.83, 75.74, 118.08, 127.30, 127.70, 128.68, 128.96, 129.74, 130.48, 132.14, 134.00, 134.59, 135.07, 135.83, 136.27, 136.78, 138.51, 146.52. Anal. RP-HPLC *Ia* (total run time 20 min): t_R = 13.04 min (purity 99%), *IIb*: t_R = 6.78 min (purity 96%). HRMS (EI) m/z calcd for $C_{27}H_{24}Cl_3N$: 469.0945 (second isotope); found: 469.0931.

4-(4-Chlorophenyl)-1-(2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-yl)piperidin-4-ol hydrochloride (1j). Yield: 61 mg (23%) of one diastereomer of **1j** as a white solid ^{1}H NMR (MeOH-d_4): δ 1.81-1.92 (m, 4H), 2.50 (br s, 1H), 2.70-3.12 (m, 5H), 3.15-3.36 (4H), 4.41-4.47 (m, 1H), 7.15-7.48 (m, 10H), 8.00 (s, 1H), 12.08 (br s, 1H). ^{13}C (MeOH-d_4): δ 35.03, 35.26, 37.25, 37.94, 38.73, 44.31, 45.84, 46.37, 69.58, 75.31, 125.81, 126.00, 127.64, 127.97, 128.67, 128.76, 129.60, 131.01, 132.58, 132.80, 133.54, 133.63, 134.80, 134.83, 144.42, 144.51. Anal. RP-HPLC *Ia* : t_R = 6.19 min (purity 100%), *IIa* (total run time 30 min): t_R = 13.45 min (purity 100%). HRMS (EI) m/z calcd for $C_{27}H_{26}Cl_3NO$: 485.1080; found:485.1073.

1-(2-(2,4-Dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-yl)-4-phenylpiperazine dihydrochloride (1l). Yield: 48 mg (22%) of of one diastereomer of **1l** as a white solid. ^{1}H NMR (MeOH-d_4): δ 2.72-2.97 (m, 3H), 3.05-3.55 (m, 8H), 3.69-3.88 (m 2H), 4.64-4.72 (m, 1H), 6.88-7.08 (m, 2H), 7.19-7.54 9m, 9H), 7.74 (d, *J* = 7.2 Hz, 1H). ^{13}C (MeOH-d_4): δ 37.56, 38.05, 40.90, 50.58, 76.79, 118.22, 122.92, 127.46, 128.73, 128.78, 129.44, 130.41, 130.51, 132.52, 133.79, 134.00, 134.66, 136.28, 136.55, 146.84, 150.63. Anal. RP-HPLC *Ia*: t_R = 7.07 min (purity 100%), *IIa*: t_R = 4.13 min (purity 98%), *IIb* (total run time 30 min): t_R = 19.11 min (purity 98%). HRMS (EI) m/z calcd for $C_{26}H_{26}Cl_2N_2$: 436.1473; found: 436.1471.

1-(2-(2,4-Dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-yl)-4-methylpiperazine dihydrochloride (1m). Yield: 105 mg (46%) of one diastereomer of **1m** as a light yellow solid. ^{1}H NMR (MeOH-d_4): δ 2.61-3.03 (m, 4H), 2.93 (s, 5H), 3.12-3.87 (m, 8H), 4.55-4.64 (m, 1H), 7.15-7.49 (m, 6H), 7.72 (d, *J* = 7.1 Hz, 1H). ^{13}C (MeOH-d_4): δ 37.59, 38.05, 40.99, 43.36, 47.18, 51.93, 77.02, 127.37, 128.72, 129.35, 130.46, 132.33, 133.98, 134.55, 136.27, 136.76, 146.61. Anal. RP-HPLC *Ia*

(total run time 20 min): t_R = 12.99 min (purity 95%), *IId*: t_R = 12.70 min (purity 96%). HRMS (EI) m/z calcd for $C_{21}H_{24}Cl_2N_2$: 374.1317; found: 374.1311.

***N*-benzyl-2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-amine fumarate (1n).** Yield: 20 mg (8%) of one diastereomer of **1n** as a white solid. ^{1}H NMR (MeOH-d_4): δ 2.69-2.79 (m, 2H), 2.89-2.95 (m, 1H), 3.02-3.08 (m, 1H), 3.17-3.25 (m, 1H), 4.14-4.21 (m, 2H), 4.62 (d, *J* = 6.1 Hz, 1H), 6.68 (s, 2H), 7.27-7.53 (m, 12H). ^{13}C NMR (MeOH-d_4): δ 32.41, 36.00, 44.64, 51.56, 65.52, 126.53, 126.60, 127.91, 128.49, 129.71, 130.01, 130.30, 130.33, 130.51, 133.42, 134.08, 135.48, 135.89, 136.00, 137.77, 144.58, 170.70. Anal. RP-HPLC *Ia*: t_R = 8.22 min (purity 100%), *IIa*: t_R = 12.37 min (purity 97%). HRMS (ESI) m/z calcd for $C_{27}H_{25}Cl_2NO_4$: 381.1051; found: 381.1046.

Pharmacology

[^{125}I]CCL5 binding experiments and [^{3}H]inositol phosphate accumulation assays were performed as previously described in the literature.[25]

References

1. Gurrath, M. Peptide-binding G protein-coupled receptors: New opportunities for drug design. *Curr. Med. Chem.* **2001**, *8*, 1605-1648.
2. Rosenkilde, M. M.; Waldhoer, M.; Lüttichau, H. R.; Schwartz, T.W. Virally encoded 7TM receptors. *Oncogene* **2001**, *20*, 1582-1593.
3. Vischer, H. F.; Vink, C.; Smit, M.J. A viral conspiracy: hijacking the chemokine system through virally encoded pirated chemokine receptors. *Curr. Top. Microbiol. Immunol.* **2006**, *303*, 121-154.
4. Proudfoot, A. E. Chemokine receptors: multifaceted therapeutic targets. *Nat. Rev. Immunol.* **2002**, *2*, 106-115.
5. Chee, M. S.; Satchwell, S. C.; Preddie, E.; Weston, K. M.; Barrel, B. G. Human cytomegalovirus encodes three G protein-coupled receptor homologues. *Nature* **1990**, *344*, 774-777.
6. Rigoutsos, I.; Novotny, J.; Huynh, T.; Chin-Bow, S. T.; Parida, L.; Platt, D.; Coleman, D.; Shenk, T. In silico pattern-based analysis of the human cytomegalovirus genome. *J. Virol.* **2003**, *77*, 4326-4344.
7. Lesniewski, M.; Das, S.; Skomorovska-Prokvolit, Y.; Wang, F.-Z.; Pellett, P. E. Primate cytomegalovirus US12 gene family: a distinct and diverse clade of seven-transmembrane proteins. *Virology* **2006**, *354*, 286-298.
8. Stassen, F. R.; Vega-Cordova, X.; Vliegen, I.; Bruggeman, C. A. Immune activation following cytomegalovirus infection: more important than direct viral effects in cardiovascular disease? *J. Clin. Virol.* **2006**, *35*, 349–353.
9. Gandhi, M. K; Khanna, R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect. Dis.* **2004**, *4*, 725-738.
10. Soderberg-Naucler, C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J. Intern. Med.* **2006**, *259*, 219-246.
11. Harkins, L.,; Volk, A. L.; Samanta, M.; Mikolaenko, I., Britt, W. J.; Bland, K. I.; Cobbs, C. S. Specific localisation of human cytomegalovirus nucleic acids and proteins in human colorectal cancer. *Lancet* **2002**, *360*, 1557–1563.
12. Cobbs, C. S.; Harkins, L.; Samanta, M.; Gillespie, G. Y.; Bharara, S.; King, P. H.; Nabors, L. B.; Cobbs, C. G.; Britt, W. J. Human cytomegalovirus infection and expression in human malignant glioma. *Cancer Res.* **2002**, *62*, 3347–3350.
13. Gao, J.-L.; Murphy, P. M. Human cytomegalovirus open reading frame US28 encodes a functional β chemokine receptor. *J. Biol. Chem.* **1994**, *269*, 28539-28542.
14. Murphy, P. M. Viral exploitation and subversion of the immune system through chemokine mimicry. *Nat. Immunol.* **2001**, *2*, 116-122.
15. Neote, K.; DiGregorio, D.; Mak, J. Y.; Horuk, R.; Schall, T. J. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. *Cell* **1993**, *72*, 415-425.
16. Kuhn, D.; Beall, C. J.; Kolattukudy, P. E. The cytomegalovirus US28 protein binds multiple CC chemokines with high affinity. *Biochem. Biophys. Res. Commun.* **1995**, *211*, 325-330.

17. Billstrom, M. A.; Lehma, L. A.; Scott Worthen, G. Depletion of extracellulair RANTES during human cytomegalovirus infection of endothelial cells. *Am. J. Respir. Cell Mol. Biol.* **1999**, *21*, 163-167.
18. Vieira, J.; Schall, T. J.; Corey, L.; Geballe, A. P. Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol*. **1998**, *72*, 8158-8165.
19. Kledal, T. N.; Rosenkilde, M. M.; Schwartz, T. W. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. *FEBS Lett.* **1998**, *441*, 209-214.
20. Streblow, D. N.; Soderberg-Naucler, C.; Vieira, J.; Smith, P.; Wakabayashi, E.; Ruchti, F.; Mattison, K.; Altschuler, Y.; Nelson, J. A. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **1999**, *99*, 511-520.
21. Pleskoff, O.; Treboute, C.; Belot, A.; Heveker, N.; Seman, M.; Alizon, M. Identification of a chemokine receptor encoded by human cytomegalovirus as a cofactor for HIV-1 entry. *Science* **1997**, *276*, 1874-1878.
22. Maussang, D.; Verzijl, D.; van Walsum, M.; Leurs, R.; Holl, J.; Pleskoff, O.; Michel, D.; van Dongen, G. A. M. S.; Smit, M. J. Human cytomegalovirus-encoded chemokine receptor US28 promotes tumorigenesis. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 13068-13073.
23. Casarosa, P.; Menge, W. M.; Minisini, R.; Otto, C.; van Heteren, J.; Jongejan, A.; Timmerman, H.; Moepps, B.; Kirchoff, F.; Mertens, T.; Smit, M. J.; Leurs, R. Identification of the first nonpeptidergic inverse agonist for a constitutively active viral-encoded G protein-coupled receptor. *J. Biol. Chem.* **2003**, *278*, 5172-5178.
24. Hulshof, J.W.; de Esch, I. J. P.; Leurs, R. Synthesis and structure-activity relationship of the first nonpeptidergic inverse agonists for the human cytomegalovirus encoded chemokine receptor US28. *J. Med. Chem.* **2005**, *48*, 6461-6471.
25. Hulshof J. W.; Vischer, H. F.; Verheij, M. H.; Fratantoni, S. A.; Smit, M. J.; de Esch, I. J. P.; Leurs, R. Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28. *Bioorg. Med. Chem.* **2006**, *14*, 7213-7230.
26. Houlihan, W. J.; Shapiro, M. J.;. Chin, J. A. Dimeric products from the base-catalyzed condensation of benzaldehyde with 2,3-dihydro-1*H*-inden-1-one or 2-(phenylmethylene)-2,3-dihydro-1*H*-inden-1-one. *J. Org. Chem.* **1997**, *62*, 1529-1531.
27. Thompson, A. S.; Humphrey, G. R.; DeMarco, A. M.; Mathre, D. J.; Grabowski, E. J. J. Direct conversion of activated alcohols to azides using diphenyl phosphorazidate. A practical alternative to Mitsunobu conditions. *J. Org. Chem.* **1993**, *58*, 5886-5888.
28. Gu, X.-H.; Yu,H.; Jacobson, A. E.; Rothman, R. B.; Dersch, C. M.; George, C.; Flippen-Anderson, J. L.; Rice, K. C. Design, synthesis, and monoamine transporter binding site affinities of methoxy derivatives of indatraline. *J. Med. Chem.* **2000**, *43*, 4868-4876.

Summary

Design and synthesis of small nonpeptidergic ligands for the human cytomegalovirus-encoded receptor US28

Several large DNA virus families, such as herpesviruses and poxviruses, have incorporated chemokine receptors from their hosts into their own viral genome to affect chemokine signaling for their own benefit. Only a few of these viral G protein-coupled receptors (GPCRs) have so far been demonstrated to be functional chemokine receptors with respect to signal transduction and chemokine binding properties. The genome of the β-herpesvirus human cytomegalovirus (HCMV) encodes several GPCRs, of which US28 is currently the most extensively characterized. Infection with HCMV has been linked to numerous pathological disorders, such as vascular diseases, transplant rejection, HIV, several inflammatory and autoimmune diseases, as well as cancer. Currently, the exact role of US28 in the pathogenesis of HCMV infection has not been completely elucidated yet, but the receptor has been suggested to play an important role in the development of e.g. vascular diseases, HIV and cancer.

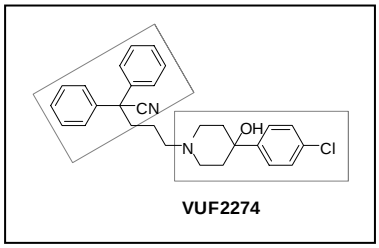
US28 is a constitutively active receptor, that signals in absence of any ligand. Blocking this viral-encoded GPCR, thereby inhibiting its constitutive signaling, could be a new strategy for the development of therapeutics against pathologies mediated by HCMV. The ligand-independent signaling of constitutively active receptors can be inhibited by inverse agonists, such as 5-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentanenitrile (VUF2274). This molecule has previously been identified as the very first small nonpeptidergic inverse agonist acting on US28.

The work described in this thesis involves the design, synthesis and pharmacological characterization of small nonpeptidergic ligands for the HCMV-encoded chemokine receptor US28. These novel ligands were used to elucidate the first structure-activity relationships on US28, resulting in a better understanding of the structural requirements that are important for ligand-

receptor interactions. This was achieved using versatile medicinal chemistry approaches, which are addressed in the different chapters of this thesis.

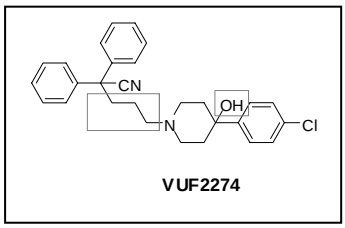
Chapter 1 gives a general introduction about the background and the context of this research. A clear survey of the potential roles of US28 is presented and we explain why we consider this receptor as a novel potential anti-viral drug target. Moreover, a detailed overview of all known US28 ligands and their structure-activity relationships (SARs) is described followed by the aim of this thesis, which is defined at the end of this chapter.

In Chapter 2 the synthesis and structure-activity relationships of the first nonpeptidergic inverse agonists for US28 are described. Starting from lead compound VUF2274, different structural modifications were introduced (see figure). Pharmacological evaluation of these ligands resulted in the very first structure-activity relationships of small nonpeptidergic ligands targeting a viral GPCR (US28) and demonstrated that a 4-phenylpiperidine moiety in the structure is essential for affinity and activity, while the nitrile group could be omitted.



VUF2274

Chapter 3 describes a new series of VUF2274 analogues, in which the rigidity is increased by the introduction of conformationally restrained tricyclic ring systems or rigid fused and nonfused piperidine ring systems. Increasing the rigidity of the propyl linker between the diphenyl group and the piperidine moiety by introduction of a double bond led to a 3-fold improvement in binding affinity compared to the lead compound. However, replacement of the 4-hydroxy group with a methylamine group was even more favourable, as this modification gave a significant 6-fold increase in affinity.



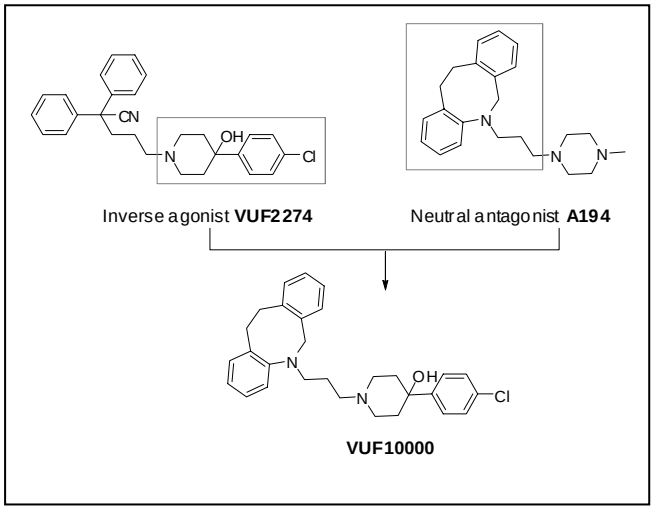
VUF2274

Although many changes were introduced in the structure of VUF2274, a more than 6-fold increase in binding affinity could not be achieved. This prompted the question whether more rigorous structural changes where needed to optimize US28 affinity. Chapter 4 describes our efforts to discover structurally diverse novel inverse agonists using molecular modelling techniques. Molecular modelling studies were initiated to design a focused virtual library around the 4-(4-chlorophenyl)piperidin-4-ol scaffold of VUF2274. Drug-like descriptors were applied to calculate a diverse subset of 50 compounds. Parallel chemistry approaches were used to synthesize these compounds and 18 ligands could easily be isolated. Next to the pharmacological evaluation of these compounds, the protocols and limitations of this drug discovery approach were discussed in Chapter 4. Pharmacological evaluation of the synthesized compounds revealed one compound as as novel inverse agonists acting on US28. All other variations attached to 4-(4-chlorophenyl)piperidin-4-ol scaffold resulted in compounds with no activity at US28.

Chapter 5 presents the results of our search for novel chemotypes targeting US28 by screening a selection of compounds from our in-house compound collection. We were aiming to discover new chemotypes for US28, because these molecules can provide new opportunities for the development of potent and selective ligands, eventually leading to promising therapeutics for clinical anti-viral intervention. The compounds from our database were selected based on the molecular similarity to structures of known ligands acting on US28. This resulted in the identification of several interesting ligands, of which some were considered as new starting points for lead optimization programs. Moreover, 5-(3-(4-methylpiperazin-1-yl)propyl)-5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine (A194) and 2-phenyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizine (B191) were disclosed as the very first small neutral antagonists acting on a viral-encoded receptor, providing us with new pharmacological tools to further investigate the pharmacological properties of US28.

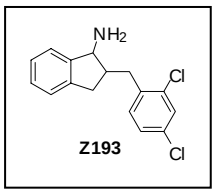
In Chapter 6, the lead optimization and structure-activity relationships of a selection of hits from the database screening is described. Interestingly, synthesis of a hybrid compound (VUF10000) containing important structural

features of inverse agonist VUF2274 and neutral antagonist A194 and subsequent pharmacological characterization of this compound showed that



replacement of the *N*-methylpiperazine group of A194 by the 4-(4-chlorophenyl)piperidin-4-ol moiety of VUF2274 (see figure) led to the anticipated recovery of the inverse agonistic properties. Thus, we have determined the molecular features that are responsible for intrinsic activity, and in particular inverse agonism versus antagonism. Moreover, these structural modifications resulted in a significant 7-fold improvement of the binding affinity compared to VUF2274 and A194. These ligands give us a better understanding of the structural requirements that are necessary for inverse agonism and neutral antagonism. Besides, the possibility to modulate the functional activity of US28 can help us to investigate the significance of constitutive US28-signaling and the role of the receptor during HCMV infection.

Screening of our in-house compound collection, as described in Chapter 5, rsulted in the identification of 2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-amine (Z193) as a novel scaffold for inverse agonism on US28. Chapter 7 describes the hit optimization of this molecule by replacing the two chloro atoms by different substituents. Moreover, different cyclic ring systems and small groups were introduced at the amine group. The synthesis and pharmacological characterization of these compounds resulted in the first structure-activity relationships for the ligand-receptor interaction of these novel inverse agonists.



Taken together, this thesis describes the design and synthesis of small nonpeptidergic ligands acting on the HCMV-encoded receptor US28 by means of multidisciplinary approaches, such as lead optimization, library screening and molecular design techniques. In this study, novel scaffolds for both inverse agonism as well as neutral antagonism have been identified. These ligands have provided us with more insights in the structural requirements that are important for binding and functional activity to US28. Ultimately, this knowledge will help us to develop compounds with an improved activity and selectivity profile on US28.

Samenvatting

Ontwerp en synthese van kleine liganden voor de door humaan cytomegalovirus gecodeerde receptor US28

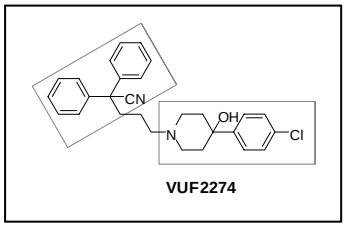
Verschillende DNA virussen, zoals herpesvirussen en pokkenvirussen, hebben chemokine receptoren van hun gastheer opgenomen in hun eigen virale genoom om het chemokine systeem van de gastheer te ondermijnen. Slechts een klein aantal van deze viraal-gecodeerde G-eiwit gekoppelde receptoren (GPCRs) zijn ook echt functionele chemokine receptoren, die in staat zijn om chemokines te binden en het 'endogene' signaal door te geven. Het genoom van het beta-herpesvirus humaan cytomegalovirus (HCMV) codeert verschillende GPCRs, waarvan US28 op dit moment het best gekarakteriseerd is. Veel mensen (50-90% van de volwassenen) zijn besmet met HCMV. Dit virus heeft alleen ernstige gevolgen voor mensen met een verzwakt immuun systeem. Onder die omstandigheden kan een infectie met het virus mogelijk bijdragen aan de ontwikkeling van hart- en vaatziektes, verschillende ontstekings- en auto-immuunziektes, kanker en HIV. Op dit moment is de exacte rol van US28 tijdens HCMV infectie niet bekend. Wel laten de uitkomsten van verschillende farmacologische onderzoeken zien dat US28 een belangrijke rol speelt binnen de virale pathofysiologie, bijvoorbeeld in de ontwikkeling van atherosclerose, HIV en kanker.

US28 is een constitutief actieve receptor, dat wil zeggen dat de receptor geactiveerd is in afwezigheid van chemokine liganden. Blokkeren van deze viraal gecodeerde receptor en het daarbij remmen van de constitutieve signalering zou een nieuwe strategie kunnen zijn voor de ontwikkeling van geneesmiddelen tegen de symptomen van HCMV infectie. De constitutieve signalering van GPCRs kan geremd worden door inverse agonisten, zoals 5-(4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl)-2,2-diphenylpentaannitrile (VUF2274). Dit molecuul is de eerste inverse agonist dat beschreven is voor US28.

Het onderzoek dat beschreven is in dit proefschrift is gericht op het ontwerp, synthese en farmacologische karakterisatie van kleine niet-peptide liganden voor de HCMV gecodeerde chemokine receptor US28. Deze nieuwe liganden zijn gebruikt om de allereerste structuur-activiteitsrelaties voor US28 liganden te defineren. Deze informatie kan vervolgens gebruikt worden om een beter inzicht te krijgen in de moleculaire aspecten van de interactie tussen de liganden en de receptor.

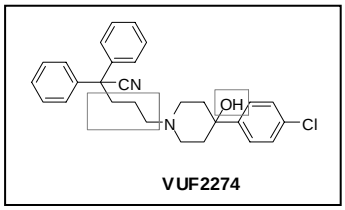
Hoofdstuk 1 geeft een algemene inleiding over de achtergronden en context waarop dit onderzoek is gebaseerd. Er wordt een overzicht van de mogelijke functies van US28 gegeven en er wordt uitgelegd waarom deze receptor beschouwd kan worden als een nieuw potentieel target voor anti-virale geneesmiddelen. Verder wordt een gedetaileerde beschrijving gegeven van alle bekende US28 liganden en hun structuur-activiteitsrelaties. Aan het einde van dit hoofdstuk wordt het doel van dit proefschrift beschreven.

In hoofdstuk 2 wordt de synthese en structuur-activiteitsrelaties van de allereerste niet-peptide inverse agonisten voor US28 beschreven. Verschillende veranderingen werden aangebracht op diverse plaatsen in de moleculaire structuur van VUF2274. Farmacologische evaluatie van deze liganden resulteerde in de allereerste structuur-activiteitsrelaties van niet-peptide liganden op een viraal gecodeerde GPCR (US28) en lieten zien dat een 4-phenyl piperidine groep in het moleuul belangrijk is voor affiniteit en activiteit, terwijl de nitril groep niet essentieel is.



VUF2274

Hoofdstuk 3 geeft een uiteenzetting van een nieuwe serie van moleculen, waarin de rigiditeit van de propyl linker tussen de diphenyl groep en de piperidine ring werd verhoogd door het invoeren van een dubbele binding. Dit resulteerde in een 3 keer hogere bindings affiniteit dan leadverbinding VUF2274. Vervanging van de 4-hydroxy groep door een methylamine groep was zelfs nog gunstiger, en



VUF2274

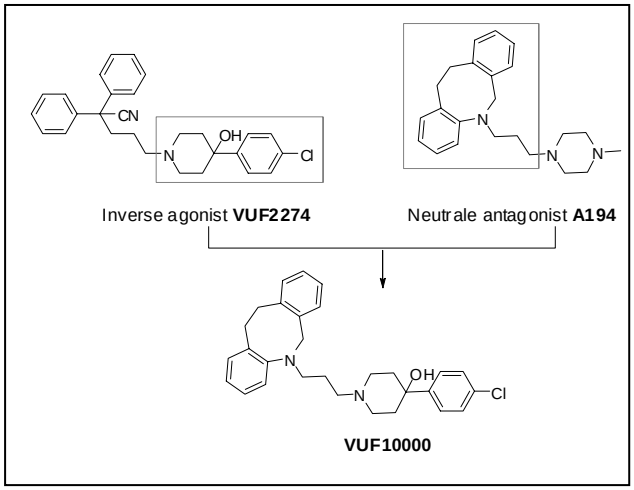
leidde tot een 6 keer hogere affiniteit.

Hoofdstuk 4 beschrijft onze pogingen om betere inverse agonisten met een verschillende structuur te identificeren met behulp van moleculaire modelling technieken. In hoofdstuk 2 en 3 worden vele derivaten van VUF2274 beschreven. Geen van deze modificaties in de structuur resulteerde in een enorme sprong in affiniteit. Daarom werd besloten om meer rigoreuze veranderen aan te brengen in de structuur. Dit werd gedaan door een virtuele bibliotheek van verbindingen te ontwerpen met als basis het commercieel verkrijgbare 4-(4-chlorophenyl)piperidin-4-ol. Uitgaande van deze verbinding en commercieel verkrijgbare reagentia werden *in silico* alle eindproducten gemaakt. Van deze virtuele verbindingen werden vele fysisch chemische eigenschappen (descriptors) berekend. Aan de hand van de gegenereerde data werd onderzocht welke (sub)set van 50 verbindingen de grootste chemische diversiteit beschreven. De geselecteerde verbindingen moesten ook voldoen aan stricte eigenschappen waaraan medicijnen in het algemeen lijken te voldoen. Vervolgens werd deze set van verbindingen gesynthetiseerd via parallele synthese technieken. Dit resulteerde in de isolatie van 18 eindproducten. Farmacologische karakterisatie van deze verbindingen resulteerde in een nieuwe inverse agonist voor US28. Alle andere gesynthetiseerde verbindingen hadden geen affiniteit of activiteit op de receptor.

In hoofdstuk 5 wordt onze zoektocht naar nieuwe chemotypes voortgezet door een database van verbindingen te screenen op US28. De moleculen werden geselecteerd op basis van de overeenkomsten die ze hebben met andere bekende US28-liganden. Het onderzoek in dit hoofdstuk leidde tot de identificatie van verschillende interessante liganden, waarvan enkelen werden gekozen als nieuw startpunt voor lead optimalisatie (zie hoofdstuk 6 en 7). Bovendien werden de twee allereerste neutrale antagonisten voor een viraal gecodeerde receptor (US28) ontdekt, namelijk 5-(3-(4-methylpiperazin-1-yl)propyl)-5,6,11,12-tetrahydrodibenzo[*b*,*f*]azocine (A194) en 2-phenyl-1,2,3,4,6,7,12,12*b*-octahydroindolo[2,3-*a*]quinolizine (B191). Deze twee

moleculen dienen als nieuwe farmacologische tools om de eigenschappen van US28 verder te onderzoeken.

Hoofdstuk 6 beschrijft de lead optimalisatie en structuur-activiteitsrelaties van een aantal verbindingen die als hits uit de database screening kwamen (zie hoofdstuk 5). Synthese van een hybride verbinding (VUF10000), die zowel belangrijke structuur eigenschappen van de inverse agonist VUF2274 als van de neutrale antagonist A194 bevat (zie figuur), en de farmacologische evaluatie liet zien that het vervangen van de *N*-methylpiperazine ring van A194 door de 4-(4-chlorophenyl)-piperidin-4-ol groep van VUF2274 leidde tot de voorziene herstel van de inverse agonistische eigenschappen. We hebben dus die moleculaire eigenschappen kunnen bepalen die verantwoordelijk zijn voor de intrinsieke activiteit, en dan voornamelijk voor inverse agonisme ten opzichte van antagonisme. Bovendien leidde deze aanpassing in de structuur tot de meest potente verbinding die ooit beschreven is op US28 met een 7 keer hogere bindingsaffiniteit dan VUF2274 en A194. Deze liganden geven een beter inzicht in de ligand-receptor interacties en de eisen waaraan de structuur van een molecuul moet voldoen voor neutraal antagonisme en inverse agonisme. Daarnaast kan de mogelijkheid om de functionele activiteit op US28 te reguleren ons helpen om het belang van de constitutieve signalering en de rol van US28 tijdens infectie met HCMV te onderzoeken.



Screening van onze eigen bibliotheek van verbindingen, zoals beschreven in hoofdstuk 5, resulteerde in de identificatie van 2-(2,4-dichlorobenzyl)-2,3-dihydro-1*H*-inden-1-amine (Z193) als een nieuwe inverse agonist voor US28.

Hoofdstuk 7 beschrijft de optimalisatie van dit molecuul door de twee chlooratomen te vervangen door andere substituenten. Daarnaast werden verschillende cyclische ring systemen en kleine groepen geïntroduceerd op het stikstofatoom. De synthese en farmacologische karakterisatie van deze verbindingen resulteerde in de eerste structuur-activiteitsrelaties van deze nieuwe groep van inverse agonisten.

Dit proefschrift heeft een beschrijving gegeven van de ontwerp en synthese van kleine liganden liganden voor de viraal gecodeerde receptor US28 via multidisciplinaire benaderingen, zoals lead optimimalisatie, screening van een bibliotheek van verbindingen en moleculaire design technieken. In dit onderzoek zijn zowel nieuwe inverse agonisten als neutrale antagonisten geïdentificeerd, die ons meer inzicht hebben geven in de eigenschappen waaraan een molecuul moet voldoen voor binding en functionele activiteit op US28. Uiteindelijk zal deze kennis ons verder helpen om liganden met een hogere activiteit en betere selectiviteit te ontwikkelen.

Curriculum Vitae

Janneke Hulshof was born on March 30, 1977 in Schagen. In 1995, she graduated from high school at the Murmellius Gymnasium in Alkmaar and started the study in Chemistry at the Vrije Universiteit (VU) of Amsterdam. She did her minor internship in Molecular Pharmacology at the Pharmacology department of the VU in the research group of Prof. Dr. Aalt Bast. Under the supervision of Dr. Frederique van Acker she investigated the anti-oxidative and toxicological activity of various synthetic flavonoids in order to find potent protectors against doxorubicin-induced cardiotoxicity. Her major research project was performed at the section Organic and Inorganic Chemistry in the group of Prof. Dr. Koop Lammertsma. Under the supervision of Dr. Jan De Wit she was involved in a research project about the synthesis and reactivity of phosphinidenes.

After her graduation in 2001, she started her PhD research in the Design and Synthesis group at the department of Medicinal Chemistry, Vrije Universiteit Amsterdam. This work was done under the supervision of Prof. Dr. Rob Leurs, Prof. Dr. Martine Smit and Dr. Iwan de Esch and resulted in this thesis. During her PhD period she was awarded the first prize for a poster at the annual NWO Medicinal Chemistry meeting in Lunteren and the first prize for an oral presentation at the Spring Symposium of the Leiden/Amsterdam Center for Drug Research, Vrije Universiteit Amsterdam. From November 2005-November 2006 she worked as a post-doctoral fellow in the same research group.
Currently, she is working as a scientific employee at the NFI (Dutch Forensic Institute) in Den Haag, performing research in the field of narcotics.

Dankwoord

Yes, mijn proefschrift is af! De vele jaren hard werken hebben uiteindelijk geleid tot het boekje wat jullie nu in handen hebben en ik ben er trots op! Vele mensen hebben me tijdens deze jaren op een of andere manier bijgestaan en bij deze wil ik jullie *allemaal* bedanken. Zonder jullie had ik het nooit gered. Een aantal mensen wil ik op deze plek graag bij name noemen.

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Voor het beoordelen van dit proefschrift wil ik de leden van de leescommissie bedanken. Ook de leden van de STW gebruikerscommissie wil ik hierbij bedanken voor de bijdrage die ze hebben geleverd aan mijn onderzoek.

Belangrijk zijn natuurlijk ook alle studenten die in meer of mindere mate hebben bijgedragen aan mijn proefschrift. Leena, thank you for the great time that we had. You were the one and only Grignard specialist, which resulted in a nice selection of compounds described in Chapter 2. Oscar en Sibel, met jullie komst was het gedaan met de rust op het lab en het was altijd heerlijk om jullie gekibbel aan te horen. Gelukkig ging dat niet ten koste van jullie werk en jullie

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