Identification of the First Nonpeptidergic Inverse Agonist for a Constitutively Active Viral-encoded G Protein-coupled Receptor*

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Human cytomegalovirus (HCMV) encodes a G protein-coupled receptor (GPCR), named US28, which shows homology to chemokine receptors and binds several chemokines with high affinity. US28 induces secretion of smooth muscle cells, a feature essential for the development of atherosclerosis, and may serve as a co-receptor for human immunodeficiency virus-type 1 entry into cells. Previously, we have shown that HCMV-encoded US28 displays constitutive activity, whereas its mammalian homologs do not. In this study we have identified a small nonpeptidergic molecule (VUF2274) that inhibits US28-mediated phospholipase C activation in transiently transfected COS-7 cells and in HCMV-infected fibroblasts. Moreover, VUF2274 inhibits US28-mediated HIV entry into cells. In addition, VUF2274 fully displaces radiolabeled RANTES (regulated on activation normal T cell expressed and secreted) binding at US28, apparently with a noncompetitive behavior. Different analogues of VUF2274 have been synthesized and pharmacologically characterized, to understand which features are important for its inverse agonistic activity. Finally, by means of mutational analysis of US28, we have identified a glutamic acid in transmembrane 7 (TM 7), which is highly conserved among chemokine receptors, as a critical residue for VUF2274 binding to US28. The identification of a full inverse agonist provides an important tool to investigate the relevance of US28 constitutive activity in viral pathogenesis.

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† The abbreviations used are: HCMV, human cytomegalovirus; GPCR, G protein-coupled receptor; RANTES, regulated on activation normal T cell expressed and secreted; HIV, human immunodeficiency virus; PLC, phospholipase C; CMV, cytomegalovirus; ORF, open reading frame; WT, wild type; HFF, human foreskin fibroblasts; InAP, inositol phosphate; TM, transmembrane.

The identification of a full inverse agonist provides an important tool to investigate the relevance of US28 constitutive activity in viral pathogenesis.

Human cytomegalovirus (HCMV) is a widespread pathogen that does not cause significant clinical manifestations in healthy individuals. In contrast, primary infection or reactivation of the virus in immunocompromised hosts, such as transplant recipients and AIDS patients, can cause severe and even fatal disease (1). Moreover, different clinical studies have suggested that HCMV infection plays a role in the development of vascular diseases including vascular allograft rejection, restenosis, and atherosclerosis (2, 3). HCMV encodes four putative GPCRs, namely US28, US29, UL33, and UL78 (4). It is interesting to notice that, like HCMV, various β- and γ-herpesviruses, such as human herpesviruses HHV-6, HHV-7, and HHV-8 (Kaposi’s sarcoma associated herpesvirus) also encode GPCRs in their genome (reviewed in Ref. 5). These virally encoded GPCRs show homology to mammalian chemokine receptors, suggesting that these viruses exploit chemokine signaling pathways as a general mechanism to interfere with the host immune system (6).

HCMV-encoded US28 shows high homology to CC-chemokine receptors and binds several CC-chemokines such as CCL5/RANTES, CCL3/macrophage inflammatory protein 1α, and CCL2/monocyte chemoattractant protein 1 with high affinity (7, 8). Furthermore, US28 binds the membrane-bound CX3C-chemokine CX3CL1/fractalkine, which has been suggested to play a role in the cellular interaction with viral particles (9). It has been demonstrated that US28, US28 displays constitutive activity, whereas its mammalian homologs do not. In this study we have identified a small nonpeptidergic molecule (VUF2274) that inhibits US28-mediated phospholipase C activation in transiently transfected COS-7 cells and in HCMV-infected fibroblasts. Moreover, VUF2274 inhibits US28-mediated HIV entry into cells. In addition, VUF2274 fully displaces radiolabeled RANTES (regulated on activation normal T cell expressed and secreted) binding at US28, apparently with a noncompetitive behavior. Different analogues of VUF2274 have been synthesized and pharmacologically characterized, to understand which features are important for its inverse agonistic activity. Finally, by means of mutational analysis of US28, we have identified a glutamic acid in transmembrane 7 (TM 7), which is highly conserved among chemokine receptors, as a critical residue for VUF2274 binding to US28. The identification of a full inverse agonist provides an important tool to investigate the relevance of US28 constitutive activity in viral pathogenesis.

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5172 This paper is available on line at http://www.jbc.org
putative new drug targets. In this study we report the identification of the first nonpeptidergic molecules able to bind a viral GPCR (US28) and to inhibit its basal signaling. These molecules provide valuable tools for the study of the role of US28 in HCMV-infected cells and serve as potential leads for the development of a new class of anti-HCMV agents.

MATERIALS AND METHODS

DNA Constructs—The cDNA encoding for US28 (12) was inserted into the mammalian vector pCDNF3 (kindly provided by Dr. J. Langer). Δ2–22-US28 and the hemagglutinin-tagged versions of both WT- and Δ2–22-US28 were generated by PCR. Single amino acid mutations, as for E277A- and E277Q-US28, were introduced using the Altered Sites™ II in vitro Mutagenesis System (Promega, Madison, WI), according to the manufacturer’s protocol. All constructs were verified by dideoxy sequencing.

Synthesis of Compounds—VUF2274 and analogues were synthesized in-house following previously published methods (16) and confirmed by NMR analysis.

Cell Culture, Transfection, and Infection with HCMV—COS-7 cells were grown as previously described (12). Transfection of the COS-7 cells was performed by DEAE-dextran, using 2 μg of DNA of each US28 construct per million cells (12). A fibroblast cell line established from human foreskin fibroblasts (HFF) was maintained in minimum essential medium with Earle’s salts (Invitrogen), 10 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and minimal essential medium nonessential amino acids (Biochrom). Cells were used between passages 14 and 16. HFFs were infected 24 h after seeding with a multiplicity of infection of 1 with the human cytomegalovirus laboratory strain AD169, or a mutant of AD169 (AD169-US28) in which the nucleotide sequence of US28 encoding residues 78 to 321 was deleted by restriction endonuclease of AD169 (AD169-US28). Cells were infected 24 h after seeding with a multiplicity of infection of 1 with the human cytomegalovirus laboratory strain AD169, or a mutant of AD169 (AD169-US28) in which the nucleotide sequence of US28 encoding residues 78 to 321 was deleted by restriction endonuclease of AD169 (AD169-US28). The HCMV strain AD169 was obtained from Dr. Ulrich H. Koszinowski, University of Munich, Munich, Germany. The AD169-US28 was constructed with the bacterial artificial chromosome (BAC) of US28 (12) and confirmed by RNA analysis.

[3H]Inositol Phosphate Production—Experiments in COS-7 cells were performed as previously described (12). 5 h after infection, HFFs were labeled by incubation in inositol-free Dulbecco’s modified Eagle’s medium supplemented with 3 μCi/ml [3H]inositol for 48 h. Subsequently, the labeling medium was aspirated, cells were washed for 10 min with Dulbecco’s modified Eagle’s medium containing 10 mM LiCl and incubated for 30 min in the same medium in the absence or presence of fractalkine (100 nM). Inositol phosphates were extracted from the cells with the chloroform/methanol method (24) and purified by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

Binding Experiments—Labeling of CCL5/RANTES (Peprotech, Rocky Hill, NJ) with [125I] and binding in COS-7 cells were performed as previously described (14). Briefly, in displacement studies transfected COS-7 cells were incubated with 0.3 nM [125I]-RANTES in binding buffer (50 mM Heps, pH 7.4, 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% bovine serum albumin) in the presence or absence of various concentrations of VUF2274 for 3 h at 4 °C. In saturation binding studies, transfected cells were incubated with different concentrations of [125I]-RANTES ranging between 0.1 and 10 nM. After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 mM NaCl. Non-specific binding was determined in the presence of 0.1 μM cold chemokine. Two days after infection, binding was performed on HFFs as described for COS-7 cells, except that 90 pM [125I]-RANTES (PerkinElmer Life Sciences) was used.

HIV-1 Infection Assay—HEK293-T cells were transiently transfected with pcDNA1-US28 and pcDNA1-CD4 using the calcium phosphate method and cultured overnight in 48-well plates. The following day, cells were incubated with different concentrations of VUF2274 (dissolved in Me2SO) or medium (containing an equal amount of Me2SO) for 2 h before infection with an R5-tropic HIV-1 virus containing the luciferase reporter gene under the long term repeat promoter. After overnight incubation, cells were washed twice and cultivated in fresh medium. 3 days after infection, the luciferase activity was quantitated using the luciferase assay kit (Promega). Enzyme-linked Immunosorbent Assay—48 h after transfection, receptor expression in COS-7 cells was measured with an enzyme-linked immunosorbent assay as previously described (14). Mouse anti-hemagglutinin monoclonal antibody (kindly provided by Dr. J. van Minnen, Vrije University, Amsterdam, The Netherlands) was used as primary antibody, and goat anti-mouse horseradish peroxidase conjugate (Bio-Rad) as secondary antibody. The TMB solution (Sigma) was used as substrate and the optical density was measured in a Victor2 (PerkinElmer Life Sciences) at 450 nm.

Toxicity Test—The AlamarBlue™ assay (Serotec, Oxford, UK) was performed following the manufacturer’s protocol. Briefly, transfected cells were incubated with VUF2274 for 2 h, followed by the addition of the AlamarBlue dye. After 1 h, the fluorescence was monitored at 560 nm excitation wavelength and 590 nm emission wavelength in a Victor2 (PerkinElmer Life Sciences).

Generation of in Silico Model of US28—An alignment was made between bovine rhodopsin and US28 using ClustalX. A homology model of US28 was generated using the homology module of Insight II, version 2.3.0 (Biosym Technologies, San Diego, CA). The A chain from the crystal structure of bovine rhodopsin was used as a template (18).

Data Analysis—Curve fitting of data was carried out using the program Prism and IC₅₀ values were obtained by nonlinear regression analysis (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± S.E.

Table I
Activities of various VUF2274 analogues

| VUF2274 | VUF5713 | VUF5715 | VUF5667 | VUF5658 | VUF5662 | VUF5660 |
|---------|---------|---------|---------|---------|---------|---------|
| IC₅₀ InsP (μM) | 3.5 | 5.3 | N.A. | 4.2 | N.A. | 14 | 29 |
| IC₅₀ Binding (μM) | 8.4 | 9.6 | N.A. | 5.9 | N.A. | 10.5 | 35 |

2 R. Minisini, C. Tulone, A. Lüsk, D. Michel, T. Mertens, P. Gierschik, and B. Moepps, submitted for publication.
Discovery of a Small Nonpeptidergic Inverse Agonist at US28—To find inverse agonists for US28, we have screened a variety of GPCR-directed ligands for modulation of the US28-mediated constitutive inositol phosphate (InsP) production. This approach has led us to identify VUF2274 (Table I) as the inverse agonist for US28. This molecule has previously been reported (16) as an antagonist for the human chemokine receptor CCR1, which shares 30% homology with US28.

VUF2274 dose dependently inhibits US28-mediated InsP production in COS-7 cells (Fig. 1A). VUF2274 inhibits ∼90% of US28 constitutive signaling with an IC50 of 3.5 μM (pIC50 = 5.46 ± 0.07; where pIC50 represents −log(IC50)). VUF2274 did not show cellular toxicity as determined with the AlamarBlue™ assay (data not shown). Moreover, other GPCRs were used as controls to ascertain specificity of action of this compound. As previously reported, expression of RCMV-encoded R33 (14) or KSHV-encoded ORF74 (19) also leads to constitutive activation of phospholipase C. VUF2274 does not modulate R33- or ORF74-mediated PLC activation at concentrations as high as 10 μM (Fig. 1B). Furthermore, VUF2274 was tested on COS-7 cells expressing the human histamine H1 receptor. Like US28, this GPCR constitutively activates PLC enzymes via Gαq/11 proteins (20). VUF2274 (10 μM) does not alter basal or histamine-induced production of InsP in COS-7 cells expressing the H1 receptor (data not shown), ruling out a possible interference with Gαq/11 proteins.

Several analogues of VUF2274 were subsequently synthesized to unravel some structural features important for its inverse agonistic effect at US28 (Table I). Variations of the benzhydryl moiety are well allowed: insertion of an ethylene (VUF5713) or thiomethylene bridge (data not shown) results in compounds of comparable potencies, indicating that some flexibility is tolerated in this part of the template. On the other hand, replacement of one of the phenyl rings with more hydrophilic moieties, such as a hydroxyl group (VUF5715), results in a loss of effect, suggesting that a bulky lipophilic moiety is important. The nitrile substituent does not appear to play a role because its removal (VUF5667) does not affect the activity of the compound. Substitution of the piperidine with a piperazine ring (VUF5658) results in an inactive compound. Finally, removal of the hydroxyl group (VUF5662) or the chlorine substituent (VUF5660) is detrimental to the inverse agonistic potency, resulting in a 5–10-fold loss in potency. A complete study of structure-activity relationships is beyond the scope of this article and will be discussed elsewhere.

Displacement of Chemokine Binding at US28—VUF2274 dose dependently displaces 125I-RANTES binding to US28 in transiently transfected COS-7 cells with an IC50 of 8.4 μM (pIC50 = 5.07 ± 0.1; Fig. 2A). Various VUF2274 analogues were also tested in 125I-RANTES displacement (Table I). In general, affinity for US28 correlates well with the potency of these compounds in the InsP assay.

We further investigated the effect of VUF2274 in a saturation binding assay of 125I-RANTES. In the presence of VUF2274, the Bmax value of 125I-RANTES is markedly decreased (Bmax = 725 ± 69 versus 380 ± 36 fmol/mg protein in the absence and presence of 10 μM VUF2274, respectively; Fig. 2B), whereas the Kd is little affected (pKd = 8.85 ± 0.01 and 8.78 ± 0.04 in the absence and presence of VUF2274, respectively), suggesting that VUF2274 acts as a noncompetitive inhibitor. In view of these results, we performed the InsP assay in the presence of both RANTES and VUF2274, to analyze the effect of chemokines on VUF2274 inhibition in a functional assay. RANTES (10–7 M), previously identified as a neutral antagonist (12), does not affect the IC50 or maximal inhibition of US28 signaling mediated by VUF2274 (pIC50 = 5.51 ± 0.1; n = 2).

Chemokines and VUF2274 Have Different Binding Pockets—To study which structural elements of US28 are responsible for interaction with chemokines and VUF2274, different mutant receptors were generated. The extracellular N-terminal domain of chemokine receptors is generally thought to play an important role in binding of chemokines (21–23). To elucidate the role of the N terminus of US28 in chemokine binding, we generated a mutant US28 receptor, which lacks the first 22 amino acids of the N terminus (referred to as Δ(2–22)-US28). To measure receptor expression in transfected cells, the WT- and Δ(2–22)-US28 receptors were epitope-tagged at their N terminus. The hemagglutinin epitope does not alter WT-US28 receptor expression, signaling, or chemokines binding (data not shown).

The N terminus deletion mutant is expressed at the cell surface (39 ± 4% compared with WT-US28, n = 3) as determined by enzyme-linked immunosorbent assay. However, Δ(2–22)-US28 does not bind any of the tested chemokines (125I-RANTES, Fig. 4A, or 125I-fractalkine, data not shown) at concentrations as high as 10 nM. Δ(2–22)-US28 still shows constitutive production of InsP (Fig. 3A), proving that truncation of the N terminus does not affect the correct orientation of the receptor.
receptor macromolecule at the cell surface. In contrast to WT-US28, Δ(2–22)-US28 basal signaling is not inhibited by the CX3C-chemokine fractalkine (Fig. 3A), again indicating the loss of chemokine binding for this mutant. Yet, VUF2274 totally inhibits the constitutive signaling of Δ(2–22)-US28 with a similar potency as observed for WT-US28 (pIC50 = 5.35 ± 0.1; Fig. 3B).

To characterize the binding site of VUF2274, an in silico model of US28 was generated based on homology with bovine rhodopsin (18). Small nonpeptidergic ligands are known to usually bind within the 7 TM domains of GPCRs (24, 25). We therefore searched for negatively charged amino acids that would be in the hydrophilic pocket between the 7 TM domains as potential interaction partners for the basic nitrogen of the piperidine moiety of VUF2274, which is predominantly protonated at physiological pH.

A glutamic acid residue in TM-7 (Glu277) appeared as an interesting candidate for its potential water accessibility. Glu277 was therefore mutated into glutamine, to eliminate the charge but retain the hydrogen bonding potential of the side chain, and into alanine, to eliminate both the charge and the hydrogen bonding potential. E277A- and E277Q-US28 are expressed at the membrane surface of COS-7 cells (Bmax = 663 ± 49; 421 ± 45 and 285 ± 57 fmol/mg of protein for WT-, E277A-, and E277Q-US28, respectively; Fig. 4A) and bind 125I-RANTES with an affinity comparable with WT-US28 (pIC50 = 8.85 ± 0.1; 8.89 ± 0.1, and 8.90 ± 0.1 for WT-, E277A-, and E277Q-US28, respectively; Fig. 4A). Interestingly, E277A- and E277Q-US28 constitutively activate PLC in a similar fashion to US28 WT (InsP levels are, respectively, 73 and 64% of US28 WT, data not shown). Taken together, these results show that the glutamic acid residue does not play a critical role for the correct organization of US28 in its active conformation and in receptor-chemokine interaction. Interestingly, the affinity of VUF2274 is markedly reduced for both of these mutants when compared with WT-US28 (Fig. 4B). These results imply that glutamate in position 277 indeed provides part of the interaction site for VUF2274, probably via an ion-pair interaction.

VUF2274 Inhibits US28-mediated Signaling in HCMV-infected Fibroblasts—Recently, some of us have reported that infection of human foreskin fibroblasts with HCMV (strain AD169) induces a consistent increase in PLC activity.2 To investigate the role of HCMV-encoded GPCR US28 in this process, a deletion mutant virus (referred to as HCMV-ΔUS28), in which the open reading frame encoding US28 has been disrupted, was generated.3 Fig. 5 shows the InsP turnover in

mock-, WT- (strain AD169), or HCMV-US28-infected HFFs. Indeed, US28 expression is mainly responsible for constitutive activation of PLC, because in HFFs infected with HCMV-ΔUS28 inositol phosphate levels are dramatically reduced (10.6 ± 1.5% of WT-HCMV-infected InsP). The CX3C-chemokine fractalkine partially inhibits (28 ± 4%) the InsP production in CMV-infected cells (Fig. 5), whereas it does not affect the InsP signaling in HCMV-ΔUS28- or mock infected HFFs. As observed with COS-7 cells, VUF2274 dose dependently inhibits US28-mediated signaling (inset, Fig. 5) in AD169-infected HFFs with an IC50 of 776 nM (pIC50 = 6.11 ± 0.04), whereas the ligand does not affect InsP levels in mock or HCMV-ΔUS28-infected cells (data not shown). As previously reported (26), US28 expressed in AD169-infected HFFs binds 125I-RANTES with high affinity. In competition binding studies VUF2274 completely displaces 125I-RANTES with an IC50 of 708 nM (pIC50 = 6.15 ± 0.1; inset, Fig. 5).

VUF2274 Inhibits US28-mediated HIV-1 Entry—US28 has been shown to be a broadly permissive co-receptor for HIV-1 when expressed in the presence of CD4 (10). To test the ability of VUF2274 to block viral entry via US28, we used a reporter gene assay. In this assay, HEK-293T cells expressing US28 and CD4 are infected by a luciferase-containing HIV-1 reporter virus. Consequently, the level of cellular luciferase activity is proportional to HIV-1 entry. Results obtained with this assay confirm that
with different concentrations of VUF2274 or Me2SO (negative control) HEK293-T cells co-transfected with CD4 and US28 were incubated are presented as relative light units (RLU). The average of three experiments, with each data point performed in triplicate, is shown. B, HEK293-T cells co-transfected with CD4 and US28 were incubated with different concentrations of VUF2274 or Me2SO (negative control) and infected with the luciferase-containing HIV-1 reporter virus. Data are presented as percentage of US28 co-receptor activity, defined as absolute increase of US28-mediated viral entry above values obtained for cells transfected with CD4 alone. The average of two experiments, with each data point performed in triplicate, is shown.

US28 shows HIV-1 co-receptor properties. Luciferase activity is increased ~20-fold in the presence of US28 when compared with cells expressing CD4 alone (fig. 6A). VUF2274 was tested at several concentrations and it showed a dose-dependent inhibitory effect of US28-mediated viral entry (Fig. 6B). At 10^{-6} M, VUF2274 reduces HIV-1 entry to 41 ± 8% of control cells. As a control, VUF2274 was tested on 293-T cells expressing CD4 and CCR5. No inhibition of viral entry was observed (data not shown), confirming the specificity of action of VUF2274.

**DISCUSSION**

Using the US28-mediated constitutive activation of PLC as a screening approach, we have identified the small nonpeptidergic molecule VUF2274 as a full inverse agonist at the HCMV-encoded chemokine receptor US28. Possible interferences of VUF2274 with G proteins or other downstream components in the InsP signaling cascade were ruled out using different GPCRs as controls. Several analogues of VUF2274 were subsequently tested. Several emerging points are noteworthy: the piperidine ring as well as the hydroxyl group and the chloro-substituent at the phenyl ring represent important moieties for the activity of VUF2274. On the other hand, a higher degree of freedom is tolerated at the benzhydryl moiety, where an increase in the lyphophilicity or the removal of the cyano group is tolerated increase in the intracellular levels of inositol phosphates. Indeed, US28 expression appears to be mostly responsible for activation of PLC, but is not the only player, as cells infected with the deletion mutant virus ΔUS28 still have inositol phosphate levels higher than mock infected cells (Fig. 5). We hypothesize that this residual activation could be mediated by one of the other GPCRs encoded by HCMV (namely US27, UL33, and UL78). At present there is increasing evidence that also UL33 displays constitutive activity in the InsP assay when transiently transfected in COS-7 cells (17) and is not affected by VUF2274. We are currently generating evidence that also UL33 displays constitutive activity in the InsP assay when transiently transfected in COS-7 cells (17) and is not affected by VUF2274. We are currently generating evidence that also UL33 displays constitutive activity in the InsP assay when transiently transfected in COS-7 cells (17).

**Fig. 6. Inhibition of US28-mediated HIV-1 entry by VUF2274.** A, HEK293-T cells were cotransfected with CD4 and US28 or CD4 alone and infected with the luciferase-containing HIV-1 reporter virus. Data are presented as relative light units (RLU). The average of three experiments, with each data point performed in triplicate, is shown. B, HEK293-T cells co-transfected with CD4 and US28 were incubated with different concentrations of VUF2274 or Me2SO (negative control) and infected with the luciferase-containing HIV-1 reporter virus. Data are presented as percentage of US28 co-receptor activity, defined as absolute increase of US28-mediated viral entry above values obtained for cells transfected with CD4 alone. The average of two experiments, with each data point performed in triplicate, is shown.

**Fig. 7. Model of VUF2274 bound to US28.** VUF2274 is accommodated within US28 TM helices (in blue). The residue Glu^{277} (in yellow) is shown interacting with the nitrogen in the piperidine moiety of VUF2274, additional potential interaction partners are shown in purple and described in the text.
Nonpeptidergic Inverse Agonists for the HCMV-encoded US28

...ating other deletion mutant viruses, such as Δ33 (lacking UL33), which will help to better understand the physiological relevance of these observations.

Infection of fibroblasts with HCMV offers a relevant model for the pharmacological study of US28, because it closely resembles the pathophysiological situation. In this condition, US28 expression is regulated by the virus and its constitutive signaling is not a potential artifact because of overexpression. In line with data obtained in COS-7 cells, VUF2274 dose dependently inhibited US28-mediated signaling in HCMV-infected fibroblasts. These results confirm the action of VUF2274 as an inverse agonist at US28 in a physiologically relevant model system. It is, however, noteworthy that maximal inhibition produced by VUF2274 on virus-infected cells is less pronounced (maximal inhibition is 70% of HCMV-induced InaP accumulation) than observed in COS-7 cells transfected with US28 receptor cDNA. This discrepancy might be because of differences in US28 expression in the two systems, or possibly because of different US28 coupling to accessory proteins in the different cell lines. Moreover, it is possible that HCMV encodes some additional signaling partner for US28 that could alter US28 behavior and that would not be present in the transfected system.

US28 is an early gene, being transcribed as early as 2 h after HCMV infection of permissive cells, such as fibroblasts (32). Consequently, cells permissive to HCMV infection express a receptor that functions in the absence of any ligand and may influence the cellular machinery early after infection. Notably, US28 constitutively activates pathways such as PLC and NF-κB, which are important for viral replication (33–35). US28 is transcribed not only in cells permissive to CMV infection, but also in latently infected cells, such as monocyes (36), suggesting that US28 may affect a wide range of cell types. In monoocytes, the transciption factor NF-κB promotes expression of over 100 target genes, mostly involved in the regulation of the immune response (37). It is suggestive to propose that US28, through activation of PLC and NF-κB pathways, might alter expression of such proteins, resulting in alteration of the immune response in favor of viral survival and spreading.

At present, the biological role of US28 in viral pathogenesis is still unclear. US28 has been suggested to act as a chemokine scavenger (38). Moreover, US28 can induce smooth muscle cell migration (11). Finally, the possible implications of the high constitutive activity of US28 in the pathogenesis of CMV infection have not been established yet. Studies conducted with a US28 deletion mutant virus have shown that US28 is not required for viral growth in culture (26), suggesting that its role is important for viral pathogenesis in vivo.

The lack of in vivo systems because of the high species specificity of HCMV makes the direct analysis of the function of US28 difficult. Generation of US28 knock-in mice might represent a good system for the analysis of this receptor. This approach has been very successful for ORF74 (39). Moreover, the recent discovery that chimneyze CMV also encodes a US28 gene (NCBI accession number NP-612800) might offer an additional animal model. The small inverse agonist VUF2274 provides a tool to investigate a potential role of US28 and its constitutive activity in activating a cell to allow or enhance viral replication in vivo.

Finally, different clinical studies (40, 41) have suggested that HCMV infection is a co-factor in HIV disease progression. In fact, HIV-positive infants CMV infection increases the chances of progression to AIDS, impaired brain growth, and motor deficits (41). Cellular entry of HIV-1 is mediated via interaction of the viral glycoprotein 120 with CD4 and a co-receptor, which belongs to the chemokine receptor family. The best characterized co-receptors are CCR5, which mediates entry of monotropic (R5) HIV strains, and CXCR4, which mediates entry of lymphotropic (X4) HIV strains. The HCMV-encoded US28 can also enhance viral entry for both R5- and X4-tropic HIV strains in vitro (10), giving a molecular basis to the epidemiological link between HCMV and HIV-1 infection. Our results with 293T cells confirm US28 as a potential co-receptor for CCR5-tropic HIV strains. The inverse agonist VUF2274 at a concentration of 1 μM inhibits viral entry by 60%, suggesting that small ligands acting at US28 might have anti-HIV properties. Further studies must be undertaken to determine in detail how VUF2274 blocks HIV-1 entry. VUF2274 might inhibit glycoprotein 120 binding at US28, or inhibit a receptor conformational change necessary for viral fusion, or alternatively induce US28 internalization. The various mechanisms have been previously suggested for different inhibitors of HIV entry that target human chemokine (co)receptors (25, 42).

Similarly, the physiological relevance of US28 as co-receptor for HIV-1 still remains to be determined. There is evidence that cellular co-infection with HIV and CMV can occur in vivo, in e.g. the brain, retina, and lungs (43, 44). CCR5 expression in the brain is very low (45), giving rise to the possibility that different co-receptors, among which US28, might serve for HIV entry and are related to the HIV-related progression of dementia.

In conclusion, we show that the small nonpeptidergic molecule VUF2274 is a full inverse agonist at the HCMV-encoded chemokine receptor US28. Moreover, VUF2274 inhibits US28-mediated HIV-1 infection. To our knowledge, this is the first example of a small inverse agonist targeted against a viral encoded GPCR. We suggest that binding of VUF2274 locks the receptor US28 in an inactive conformation and allosterically modulates chemokine binding at US28. The identification of an inverse agonist provides a tool for further dissecting the role of US28 and its constitutive activity in HCMV infection. In addition, it might serve as a potential lead for innovative antiviral drug design.

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