Agonists and Inverse Agonists for the Herpesvirus 8-encoded Constitutively Active Seven-transmembrane Oncogene Product, ORF-74*

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A number of CXC chemokines competed with similar, nanomolar affinity against 125I-interleukin-8 (IL-8) binding to ORF-74, a constitutively active seven-transmembrane receptor encoded by human herpesvirus 8. However, in competition against 125I-labeled growth-related oncogene (GRO)-α, the ORF-74 receptor was highly selective for GRO peptides, with IL-8 being 10,000-fold less potent. The constitutive stimulating activity of ORF-74 on phosphatidylinositol turnover was not influenced by, for example, IL-8 binding. In contrast, GRO peptides acted as potent agonists in stimulating ORF-74 signaling, whereas IP-10 and stromal cell-derived factor-1α surprisingly acted as inverse agonists. These peptides had similar pharmacological properties with regard to enhancing or inhibiting, respectively, the stimulatory effect of ORF-74 on NIH-3T3 cell proliferation. Construction of a high affinity zinc switch through introduction of two His residues at the extracellular end of transmembrane segment V enabled Zn2⁺ to act as a prototype non-peptide inverse agonist, which eliminated the constitutive signaling. It is concluded that ORF-74, which is believed to be causally involved in the formation of highly vascularized tumors, has been optimized for agonist and inverse agonist modulation by the endogenous angiogenic GRO peptides and angiostatic IP-10 and stromal cell-derived factor-1α, respectively. ORF-74 could serve as a target for the development of non-peptide inverse agonist drugs as demonstrated by the effect of Zn²⁺ on the metal ion site-engineered receptor.

Chemokines are chemotactic cytokines that ensure that leukocytes migrate to the right tissue or compartment at the right time. During inflammation, chemokines are also involved, for example, in the extravasation process and recruitment of the appropriate type of leukocytes to the infected tissue. Furthermore, chemokines are involved in cellular communication controlling processes such as angiogenesis (1, 2). In a number of herpesviruses and poxviruses, genes coding for homologs of chemokines as well as chemokine receptors have been found (3, 4). Conceivably, these molecules have been obtained by the virus through an ancient act of molecular piracy and subse-

quentely are structurally optimized for a particular pharmacological phenotype of benefit to the virus. In the case of several of the virally encoded chemokine ligands, their purpose appears to be rather obvious since they act as broad spectrum chemokine antagonists, which could be used by the virus to prevent the local recruitment of leukocytes (5, 6). However, it is still rather unclear what the function of the virally encoded chemokine receptors is. In general, these receptors are not required for virus replication in vitro (7). Yet, gene deletion experiments in both mouse and rat cytomegaloviruses have shown that, for example, the UL33 receptor, which is homologous to the U12 receptor encoded by human herpesvirus 6 and 7, is essential for targeting and/or replication of the virus in salivary glands (8, 9). More important, the viral strains in which the UL33 receptor was specifically knocked out were less virulent than wild-type cytomegalovirus as judged from survival of infected animals (9).

ORF-74 is a CXC chemokine receptor encoded by many γ-herpesviruses, including the recently discovered human herpesvirus 8 (HHV8) or Kaposi’s sarcoma-associated herpesvirus (see Fig. 1) (10, 11). Initially, the ORF-74 gene product from Herpesvirus saimiri, ECRF3, was shown to bind interleukin-8 (IL-8) with high affinity even though it structurally is only distantly related to the mammalian IL-8 receptors, CXCR-1 and CXCR-2 (12). Interestingly, the ORF-74 receptor from HHV8 was found to be highly constitutively active (13). Furthermore, although IL-8 binds with high affinity to ORF-74 from HHV8, it does not affect the signaling of the receptor (13). In contrast to other mammalian chemokine receptors, which preferentially signal through the Gαi pathway, ORF-74 activates the phospholipase C pathway, leading to constitutively high turnover of phosphatidylinositol, as well as signals through the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase, leading to the production and secretion of vascular endothelial growth factor (14). As a result of these activities, ORF-74 functions as an oncogene, leading to cellular transformation and development of highly vascularized tumors in nude and SCID mice (15). Consequently, it has been proposed that ORF-74 could be causally involved in the development of Kaposi’s sarcoma lesions and lymphomas associated with HHV8 infection (14, 15). Since ORF-74 belongs to the class of rhodopsin-like seven-transmembrane receptors, which classically are good drug targets, it appears that it should be possible

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¶ The abbreviations used are: HHV8, human herpesvirus 8; CXCR, CXC receptor; IL-8, interleukin-8; vMIP-II, viral macrophage inflammatory protein II; GRO, growth-related oncogene; IP-10, interferon γ-inducible protein; SDF-1α, stromal cell-derived factor-1α; ENA-78, epithelial cell-derived activating peptide-78; NAP-2, neutrophil-activating peptide-2; MIP, macrophage inflammatory protein; MCP, monocyte chemotactic protein.

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The ORF-74 receptor was transiently transfected in COS-7 cells, and competition binding and phosphatidylinositol turnover experiments were performed as described under “Experimental Procedures.” IC_{50} values determined in competition either against 125I-IL-8 (B_{max} = 42 ± 12 fmol/10^5 cell) or against 125I-GRO{alpha} (B_{max} = 4.4 ± 10 fmol/10^5 cell) are shown as means ± S.E. of the indicated number of experiments (n). The fold-difference in affinity using 125I-GRO{alpha} versus 125I-IL-8 as radioactive ligand is listed. EC_{50} values for the individual chemokines in either stimulating (S) or inhibiting (I) the basal constitutive activity of ORF-74 as determined by phosphatidylinositol turnover are shown as means ± S.E. of the indicated number of experiments (n).

### Agonists and Inverse Agonists for ORF-74

| Chemokine     | Competition binding  | -Fold difference | PP turnover, basal activity |
|---------------|----------------------|------------------|-----------------------------|
|               | 125I-IL-8 IC_{50} nm | 125I-GRO{alpha} IC_{50} nm |                       |
| GRO{alpha}    | 0.23 ± 0.1 (5)       | 0.10 ± 0.02 (11) | 0.4                        | 1.1 ± 0.2 (9) | S                      |
| GRO{beta}     | 0.34 ± 0.1 (5)       | 0.22 ± 0.05 (5)  | 0.7                        | 3.1 ± 1.1 (5) | S                      |
| GRO{gamma}    | 0.37 ± 0.1 (5)       | 0.06 ± 0.01 (5)  | 0.2                        | 2.8 ± 0.8 (4) | S                      |
| NAP-2         | 3.6 ± 0.6 (4)        | 82 ± 12 (5)      | 23                         | >1000 (6)    |                       |
| IL-8          | 1.5 ± 0.4 (5)        | 1330 ± 800 (5)   | 890                        | >1000 (2)    | >1000 (2)             |
| ENA-78        | 11 ± 2.9 (2)         | 173 ± 31 (3)     | 16                         | 604 ± 309 (6)| S                      |
| IP-10         | 4.2 ± 1.1 (3)        | 2.3 ± 0.6 (3)    | 0.6                        | 3.3 ± 0.3 (3)| I                      |
| SDF-1a        | 6.7 ± 1.5 (5)        | 13 ± 3 (4)       | 2                          | 11 ± 3.8 (5)| I                      |
| Met-SDF-1a    | 68 ± 21 (3)          | 30 ± 3.6 (5)     | 0.4                        | 28 ± 6.1 (5)| I                      |
| vMIP-II       | 38 ± 7.6 (7)         | 72 ± 8.7 (7)     | 1.9                        | 84 ± 20 (6) | I                      |

* PI, phosphatidylinositol.

### Experimental Procedures

**Materials—** The human chemokines were purchased from Peprotech (IL-8, GRO{alpha}, and GRO-1) or R&D Systems (GRO{beta}, GRO{gamma}, ENA-78, and IP-10) or were kindly provided by Timothy N. C. Wells (Serono, Geneva, Switzerland; NAP-2 and vMIP-II) or Michael A. Luther (Glaxo Wellcome; Met-SDF-1a). ORF-74 (GenBankTM accession number U24275) was cloned from a biopsy taken from a Kaposi’s sarcoma skin lesion from an human immunodeficiency virus type 1-infected patient (5). The cDNA was cloned into the eukaryotic expression vector pETE8 (16). Monoiodinated 125I-IL-8, 125I-GRO{alpha}, and myo-[3H]inositol (Perkin-Elmer Life Sciences) were purchased from Amersham International (Buckinghamshire, United Kingdom). AG 1-X8 anion-exchange resin was from Bio-Rad.

**Transfections and Tissue Culture—** COS-7 cells were grown at 10% CO_2 and 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.01 mg/ml gentamicin.

**Binding Experiments—** COS-7 cells were transfected to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the individual clones; the goal was to obtain 5–10% specific binding of the added radioactive ligand. Two days after transfection, cells were assayed by competition binding performed on whole cells for 3 h at 4 °C using 12 pm 125I-IL-8 or 125I-GRO{alpha} plus variable amounts of unlabeled ligand in 0.5 ml of 50 mM Hepes buffer, pH 7.4, supplemented with 1 mM CaCl_2, 0.5% (w/v) bovine serum albumin, and were incubated at 37 °C for 30 min. The resulting supernatant was neutralized with KOH in Hepes buffer, and the generated [3H]inositol phosphates were purified on AG 1-X8 anion-exchange resin (18). Determinations were made in duplicate.

**Cell Proliferation Assay—** Receptor selection and amplification technology assays (R-SATTM, Acadia Pharmaceuticals Inc., San Diego, CA) were performed as described previously (19). NIH-3T3 cells (7.5 × 10^5) were plated 1 day before transfection into 10-cm Petri dishes and transfected with 0.3 µg of receptor DNA (ORF-74) in the pTEJ8 vector and 4 µg of pSIC-β-galactosidase DNA (Promega). One day after transfection, the cells were split into 96-well plates, and ligands were added the following day. After 24 h of exposure to ligand, cell proliferation was quantified using a standard colorimetric assay for β-galactosidase activity as described previously (19, 20).

**IC_{50} and EC_{50} values were determined by nonlinear regression, and B_{max} values were calculated using Inplot Version 4.0 software (GraphPAD Software for Science, San Diego).**

### RESULTS

ORF-74 was cloned from a Kaposi’s sarcoma skin lesion. Only a single silent G-to-T nucleotide mutation was identified at position 907 in the coding region of ORF-74 as compared with the sequence deposited in GenBankTM (accession number U24275). This represents a very high degree of conservation in comparison with, for example, the US28 receptor from human cytomegalovirus, which is widely divergent in nucleotide and amino acid sequences among different viral strains (21).

**Binding Experiments—** Using 125I-IL-8 as tracer in competition binding experiments in COS-7 cells transiently expressing ORF-74, we could confirm that this virally encoded receptor binds a number of human CXC chemokines with high and rather similar affinity (Table I and Fig. 2, A–C). Even the structurally rather distantly related chemokines IP-10 and SDF-1a (Fig. 1) could displace 125I-IL-8 with single-digit nanomolar affinity. In contrast, the CC chemokines MIP-1{alpha}, MIP-1{beta}, MCP-1, and RANTES (regulated on activation, normal T-cell expressed and secreted) bound to the receptor with affinities (IC_{50} < 10,000 nM (n = 3), whereas MCP-3 and aminoxyxepentane (AOP)-RANTES had measurable affinities around 200 nM (n = 3) when tested against IL-8 as radioactive ligand (data not shown).
Previously, only IL-8 has been employed as a radioactive ligand in binding experiments with ORF-74 (13). However, since GROα displaced 125I-IL-8 with even higher affinity (IC<sub>50</sub> = 0.23 nM) than unlabeled IL-8 itself (IC<sub>50</sub> = 1.5 nM), we decided to probe ORF-74 with 125I-GROα as well. As shown in Fig. 2 (D–F), the ligand binding profiles displayed by 125I-GROα were different from those displayed by 125I-IL-8, in particular for the ligands ENA-78, NAP-2, and IL-8 (Fig. 2, B versus E). The competition binding curves for these three ligands were shifted 16-, 23-, and 890-fold to the right, respectively, as compared with the binding curves observed in competition with 125I-IL-8. In contrast, the competition binding curves for GROα, -β, and -γ were shifted 2–5-fold to the left in competition with 125I-IL-8, in particular for the ligands ENA-78, NAP-2, and IL-8 (Fig. 2, A versus D). The binding curves for IP-10, SDF-1a, and vMIP-II were rather similar in both assays (Fig. 2, C and F). In conclusion, with 125I-GROα as tracer, ORF-74 appears to have ≥20 times higher affinity for the three GRO peptides than for IP-10 and 100 to >1000 times higher affinity than for any other chemokine ligand, with SDF-1a being the closest one. Thus, the affinity of ORF-74 for the GRO peptides is ~10,000-fold higher than the affinity for IL-8 in competition with radioactive GROα (Table I).

**Signal Transduction Analysis**—The reported ability of ORF-74 to stimulate phosphatidylinositol turnover in a ligand-independent manner was confirmed by gene dosage experiments in transiently transfected COS-7 cells (Fig. 3) (13). The inability of IL-8 to affect this constitutive signaling was also confirmed (Fig. 4B) (13). However, surprisingly, it was found that in contrast to IL-8, GROα and GROγ were able to stimulate ORF-74 signaling >2-fold and with EC<sub>50</sub> values of 1.1 and 2.8 nM, respectively (Fig. 4A). GROβ also acted as an agonist, yet only with a partial response as compared with GROα, but with a similar EC<sub>50</sub> value of 3.1 nM. Like IL-8, NAP-2 and ENA-78 were unable to affect signaling through ORF-74 except at micromolar concentrations, where NAP-2 stimulated the phosphatidylinositol turnover to ~50% of the maximal response observed with GROα (Fig. 4B). Most surprisingly, IP-10, SDF-1a, and Met-SDF-1a functioned as efficient inverse agonists on ORF-74 with potencies very similar to their binding affinities measured against GROα as radioligand: EC<sub>50</sub> = 3.3, 11, and 28 nM, respectively (Fig. 4C), versus affinity = 2.3, 13, and 30 nM, respectively (Table I). vMIP-II, which like ORF-74 is encoded by HHV8 and which acts as an antagonist on multiple human chemokine receptors (5), could also block the constitutive signaling of ORF-74, albeit only with an EC<sub>50</sub> of 84 nM; however, this potency was similar to the affinity (IC<sub>50</sub> = 72 nM) for vMIP-II measured against GROα as radioligand. When ORF-74 signaling was stimulated with 10 nM GROα, the four inverse agonists (IP-10, SDF-1a, Met-SDF-1a, and vMIP-II)
acted as antagonists (Fig. 4F). Neither of the other chemokines had any effect on the stimulated activity (Fig. 4, D and E).

**Cell Transformation Assay.—** Cotransfection of NIH-3T3 cells with ORF-74 and the marker enzyme β-galactosidase was used to measure effects on cell transformation using the R-SAT™ assay (19). In agreement with previously published data showing an effect on foci formation (13), ORF-74 was found to strongly stimulate cell proliferation in a ligand-independent manner as determined by β-galactosidase activity in the transfected cells. The constitutive activity of ORF-74 resulted in a β-galactosidase activity (corresponding to degree of cell transformation) that was stronger than observed with, for example, the muscarinic m1 receptor after maximal ligand stimulation.

**Inhibition of ORF-74 Signaling by "Non-peptide" Zinc Ions—**

Through the introduction of His residues, we have previously both structurally and functionally exchanged a non-peptide antagonist-binding site in the tachykinin NK1 receptor with metal ion sites (22, 23). In contrast to binding sites for peptides and non-peptide ligands, these metal ion sites can be transferred even to distantly related seven-transmembrane receptors (24). To probe the susceptibility of ORF-74 to inhibition by non-peptide inverse agonists, two His residues were introduced at positions V:01 (Arg208) and V:05 (Arg212), corresponding to two of the positions previously tested in both the NK1 and κ-opioid receptors (Fig. 1). Binding of 125I-IL-8 was eliminated in the R208H,R212H ORF-74 mutant receptor. This is in accord with the fact that the two Arg residues, which were substituted with His residues, are conserved between the ORF-74 and endogenous CXCR-1 and CXCR-2 receptors, in which these two residues previously have been shown to be involved in IL-8 binding (25). However, although the mutant receptor did not bind IL-8, it was nevertheless expressed well and still displayed a high degree of constitutive signaling (Fig. 3).

In the R208H,R212H ORF-74 mutant receptor, Zn2+ acted as a potent inverse agonist in blocking the constitutive signaling, with an EC50 of ~1 μM (Fig. 6). Thus, the effect of this "prototype" non-peptide antagonist (Zn2+; used here in a zinc switch on the mutant receptor) indicates that it should be possible to develop non-peptide inverse agonists targeted toward the extracellular part of ORF-74.

**DISCUSSION**

This study indicates that the ORF-74 receptor encoded by HHV8 has been optimized by the virus to recognize GRO peptides from its host as agonistic modulators of its high constitutive activity. Inhibition of this constitutive activity in wild-type ORF-74 by the chemokines IP-10 and SDF-1α and especially in the zinc site-engineered mutant receptor by Zn2+ indicates that this viral oncoprotein is susceptible to drug intervention by non-peptide inverse agonists.

**Chemokine Ligands for ORF-74—** Although ORF-74 can be shown to bind other CXC chemokines such as IL-8, NAP-2, and ENA-78 with nanomolar affinity in binding assays employing 125I-IL-8, these "non-GRO" chemokines are in fact at physiological concentrations not able to interfere with either GROα binding or ORF-74 signaling. The only exceptions are IP-10 and SDF-1α, which, at nanomolar concentrations, function as efficient inverse agonists in ORF-74 signaling. It is interesting to note that the affinities measured in competition against 125I-GROα for all the chemokines appear to closely reflect their potency upon receptor signaling (Table I; compare Fig. 2 (A–C) with Fig. 4 (A–C)). For the agonists, the EC50 values were shifted ~10-fold to the right, as compared with their GROα affinity. Thus, the GRO peptides show affinities around 0.1 nM in competition with GROα, and they stimulate signaling with an EC50 of ~1 nM. NAP-2 has an affinity against GROα binding or ORF-74 signaling. The only exceptions are IP-10 and SDF-1α, which, at nanomolar concentrations, function as efficient inverse agonists in ORF-74 signaling. It is interesting to note that the affinities measured in competition against 125I-

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tion of the receptor. In contrast, there is no functional correlate to the single-digit nanomolar affinity for IL-8, NAP-2, and ENA-78 as measured in competition with \(^{125}\)I-IL-8. Apparently, at physiological concentrations, these peptides merely function as neutral ligands on the ORF-74 receptor.

The ORF-74 gene product from Herpesvirus saimiri was originally described as a viral IL-8 receptor (12). However, in fact, ECRF3 also binds GRO\(^{\alpha}\) preferentially as described here for ORF-74 from HHV8. There is a 50-fold difference in favor of GRO\(^{\alpha}\) versus IL-8 with respect to potency for stimulating calcium mobilization through ECRF3. In contrast, on human CXCR-2, there is 300-fold difference in favor of IL-8 versus GRO\(^{\alpha}\) (12). Thus, it appears that ORF-74 receptors from \(g\)-herpesvirus in general have been optimized to recognize GRO peptides.

The phenomenon that ligands (exemplified here by IL-8) can bind to a seven-transmembrane receptor with high affinity without being able to compete for binding against other ligands has been extensively studied, especially in the tachykinin and opioid receptor systems (17, 26–29). In the chemokine system, this phenomenon has previously been reported in CXCR-2, where, for example, the affinity for NAP-2 can vary up to 2000-fold depending upon whether it is measured against \(^{125}\)I-ENA-78 (0.5 nM) or against \(^{125}\)I-IL-8 (1 \(\mu\)M) (30). The molecular or cell biological correlate to this general phenomenon is, however, not yet clear (29). The most simple explanation would be that the ligands bind to two (or more) different sites on the receptor. This is, however, hard to imagine with the relatively large chemokine ligands of this study, although it is not impossible. Another explanation could be that ligands can bind to two (or more) distinct conformations of the receptor that do not interchange readily (29). These conformations of states could represent, for example, complexes with different G-protein subtypes and/or monomeric versus dimeric forms of the receptor. Whatever the molecular basis is, it is nevertheless a phenomenon that has been exploited by viruses not only in the ORF-74 system, but also in other virally encoded chemokine receptors such as US28 from human cytomegalovirus, possibly to obtain selective recognition in systems where multiple ligands are

**FIG. 4.** Effect of selected chemokines on basal (A–C) and GRO\(^{\alpha}\) (10 \(\times\) 10\(^{-8}\) M)-stimulated (D–F) phosphatidylinositol turnover induced by ORF-74. Experiments were performed on transiently transfected COS-7 cells as described in detail under “Experimental Procedures.” A and D, GRO\(^{\alpha}\) (●) \((n = 9)\), GRO\(^{\beta}\) (▼) \((n = 5)\), and GRO\(^{\gamma}\) (□) \((n = 4)\). B and E, IL-8 (■) \((n = 11)\), NAP-2 (▼) \((n = 6)\), and ENA-78 (●) \((n = 4)\). C and F, IP-10 (▲) \((n = 3)\), SDF-1\(^{\alpha}\) (□) \((n = 5)\), Met-SDF-1\(^{\alpha}\) (□) \((n = 5)\), and vMIP-II (△) \((n = 6)\). PI, phosphatidylinositol.

**FIG. 5.** Chemokine modulation of constitutive ORF-74-induced cell transformation. NIH-3T3 cells were cotransfected with the ORF-74 receptor and the marker gene β-galactosidase, and the assay was performed as described in detail under “Experimental Procedures.” ●, GRO\(^{\alpha}\); ▲, IP-10; □, Met-SDF-1\(^{\alpha}\).

**FIG. 6.** Inhibition by Zn\(^{2+}\) of constitutive phosphatidylinositol turnover induced by ORF-74 with an engineered bis-His metal ion switch. ●, wild-type ORF-74 (ORF74 wt) \((n = 5)\); ▲, R208H,R212H ORF-74 \((n = 7)\). In the mutant receptor, His(V:01) (His\(^{208}\)) and His(V:05) (His\(^{212}\)) are located in \(i\) and \(i+4\) positions, which in a helical configuration is optimal for binding zinc ions (see Fig. 1). PI, phosphatidylinositol.
Modulation of Constitutive ORF-74 Signaling—The observation that ORF-74 is stimulated by GRO peptides and inhibited by IP-10 and SDF-1α is interesting since these peptides are generally perceived to be angiogenic and angiostatic factors, respectively (31). In addition to its cell transforming properties, ORF-74 has, through its induction of expression of vascular endothelial growth factor, been strongly implicated in the angiogenesis involved in the formation of Kaposi’s sarcoma (15). The angiogenic activity of GROα and the angiostatic activity of IP-10 have been demonstrated in several systems, both in vitro (endothelial chemotaxis) and in vivo (neovascularization and tumor formation) (31–34). SDF-1α or rather its endogenous receptor (CXCR-4) was recently, through gene knockout experiments, shown to be important for the remodeling part of the neovascularization process, i.e., the reorganization of small immature vessels into larger mature vessels (2). Thus, according to the present data, the virally encoded ORF-74 receptor apparently exploits endogenous ligands, which normally are involved in angiogenesis or neovascularization in the host organism, for both positive and negative modulation of its high constitutive activity.

Due to its oncogenic properties and its effect on neovascularization, ORF-74 appears to be an interesting drug target for the treatment of HHV8-associated malignancies. Seven-transmembrane receptors are in general excellent drug targets, and non-peptide antagonists have accordingly been developed for a number of neuropeptide and peptide hormone receptors (35, 36). The first high affinity non-peptide antagonists for also chemokine receptors have recently been reported (37–39). However, since we do not yet know the structural reason for the high constitutive activity of the ORF-74 receptor, it is far from evident that it should be possible to stop its signaling by binding of a small non-peptide ligand to its extracellular surface. Although there is general agreement that the binding sites for non-peptide ligands usually are significantly different from those for the endogenous peptide ligands (35, 40, 41), the molecular mechanism of action of non-peptide ligands is the subject of debate. One opinion is that the non-peptide antagonists act by blocking the binding of the agonist through a space-filling process, although their binding sites may not overlap directly (42). Another view is that the antagonist and agonist bind to sites presented by different conformations and that the ligands compete for the whole receptor in an allosteric system (35). Thus, in the original report on zinc site engineering, we suggested that Zn$^{2+}$ acted as an allosteric antagonist that stabilized an inactive conformation, which thereby prevented the receptor from going into the active conformation (which would bind the agonist) rather than actually directly interfering with the binding of the ligand (22). This suggestion fits with the observations of the present study that the metal ion binding at this location inhibits the ligand-independent signaling. From a drug development point of view, the inhibition of ORF-74 signaling by Zn$^{2+}$ in the mutant receptor can be considered as proof of the concept that non-peptide compounds that can block the constitutive signaling of this viral oncosite can be developed, even though they target the extracellular part of the receptor.