HIV Integrase Inhibitors Block Replication of Alpha-, Beta-, and Gammaherpesviruses

Zhipeng Yan, Kevin F. Bryant, Sean M. Gregory, Magdalena Angelova, David H. Dreyfus, Xue Zhi Zhao, Donald M. Coen, Terrence R. Burke, David M. Knipe

Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA; Allergy and Clinical Immunology/Pediatrics, Yale School of Medicine, New Haven, Connecticut, USA; Chemical Biology Laboratory, Frederick National Laboratory for Cancer Research, Center for Cancer Research, National Cancer Institute—Frederick, National Institutes of Health, Frederick, Maryland, USA; Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

The catalytic site of the HIV integrase is contained within an RNase H-like fold, and numerous drugs have been developed that bind to this site and inhibit its activity. Herpes simplex virus (HSV) encodes two proteins with potential RNase H-like folds, the infected cell protein 8 (ICP8) DNA-binding protein, which is necessary for viral DNA replication and exhibits recombination activity in vitro, and the viral terminase, which is essential for viral DNA cleavage and packaging. Therefore, we hypothesized that HIV integrase inhibitors might also inhibit HSV replication by targeting ICP8 and/or the terminase. To test this, we evaluated the effect of 118-D-24, a potent HIV integrase inhibitor, on HSV replication. We found that 118-D-24 inhibited HSV-1 replication in cell culture at submillimolar concentrations. To identify more potent inhibitors of HSV replication, we screened a panel of integrase inhibitors, and one compound with greater anti-HSV-1 activity, XZ45, was chosen for further analysis. XZ45 significantly inhibited HSV-1 and HSV-2 replication in different cell types, with 50% inhibitory concentrations that were approximately 1 μM, but exhibited low cytotoxicity, with a 50% cytotoxic concentration greater than 500 μM. XZ45 blocked HSV viral DNA replication and late gene expression. XZ45 also inhibited viral recombination in infected cells and ICP8 recombinase activity in vitro. Furthermore, XZ45 inhibited human cytomegalovirus replication and induction of Kaposi’s sarcoma herpesvirus from latent infection. Our results argue that inhibitors of enzymes with RNase H-like folds may represent a general antiviral strategy, which is useful not only against HIV but also against herpesviruses.

Importance

The herpesviruses cause considerable morbidity and mortality. Nucleoside analogs have served as effective antiviral agents against the herpesviruses, but resistance can arise through viral mutation. Second-line anti-herpes drugs have limitations in terms of pharmacokinetic properties and/or toxicity, so there is a great need for additional drugs for treatment of herpesviral infections. This study showed that the HIV integrase inhibitors also block herpesviral infection, raising the important potential of a new class of anti-herpes drugs and the prospect of drugs that combat both HIV and the herpesviruses.
have been shown or are predicted to contain RNase H-like folds: the large subunit of the viral terminase, which in HSV is named the pUL15 protein and in human cytomegalovirus (HCMV) the pUL89 protein (9, 10), and the single-stranded DNA (ssDNA)-binding protein, which in HSV is named infected cell protein 8 (ICP8) or the UL29 gene product (11). In the viral terminase subunit, the carboxy-terminal domain of the HCMV pUL89 protein shows an RNase H-like fold in its crystal structure, the protein has a nuclease activity that is dependent on Mn2+ ions, and the enzymatic activity is inhibited by raltegravir (10). Similarly, the C-terminal domain of the HSV-1 terminase large subunit, pUL15, shows an RNase H-like fold in its crystal structure and shows metal ion-catalyzed cleavage of DNA (9). ICP8 is predicted to have an RNase H-like fold containing DDE residues characteristic of a metal-binding site (11, 12). ICP8 promotes strand invasion in an in vitro assay to form a displacement loop, or D loop (13), and cooperates with the HSV-1 U12 nuclease to mediate strand exchange between a long double-stranded linear molecule and a circular ssDNA molecule (14). Because ICP8 (15) and pUL15 (16) are essential for HSV replication, we tested whether HIV integrase inhibitors would inhibit HSV replication. We found that IN inhibitor compounds reduce HSV-1, HSV-2, and HCMV replication, as well as reactivation of KSHV from latent infection in cell culture.

RESULTS

We tested whether IN inhibitors could inhibit HSV replication by incubating HSV-1-infected cells in the presence of the IN inhibitors raltegravir (17) and 118-D-24 (18) and measuring the yield of progeny virus by plaque assay on new cells. We observed that raltegravir (results not shown) and 118-D-24 (see Fig. S1 in the supplemental material) inhibited HSV-1 strain KOS replication with a submillimolar 50% effective concentration (EC50) (Fig. 1). We further screened a series of related integrase inhibitor compounds (see Table S1 in the supplemental material), and these compounds showed various levels of inhibition of HSV replication (see Fig. S1). Several hydrazide compounds, XZ45, XZ15, XZ49, and XZ48, showed strong inhibition of HSV replication, reducing viral yields by five to seven orders of magnitude. In addition, two of the oxoisoindoles, XZ100 and XZ99, inhibited HSV replication by at least seven orders of magnitude (see Fig. S1).

More complete dose-response curves using the six most effective compounds and, as a control, the less efficacious compound XZ50 showed that XZ45 was the most potent (Fig. 2), so it was chosen for further study of its effects on HSV replication and cytotoxicity. XZ45 inhibited HSV-1 KOS replication in HEp-2 cells with an EC50 of 1.1 μM (Fig. 3A) and in human foreskin fibroblasts (HFFs) with an EC50 of 350 nM (Fig. 3B). In contrast, the compound showed a 50% cytotoxic concentration (CC50) of 570 μM in HEp-2 cells using the Cell Titer-Glo assay (Fig. 3C). To determine the breadth of the effects of XZ45 against HSV, we tested its effect on other HSV-1 and HSV-2 strains. XZ45 was also potent and efficacious for inhibition of replication of the limited-passage HSV-1 F and HSV-2 G strains (Fig. 3D).

To determine the stage(s) at which XZ45 inhibited HSV-1 replication, we examined viral protein synthesis in HEp-2 cells infected with HSV-1 KOS virus in the presence or absence of 10 μM XZ45. Expression and accumulation of the immediate-early ICP27 protein and the early ICP8 protein were not affected through 12 hpi (Fig. 4A). However, expression of the late glycoprotein C (gC) was inhibited (Fig. 4A). Therefore, viral replication was inhibited after E gene expression and at or before L gene expression. We therefore hypothesized that XZ45 inhibited viral DNA replication. In HEp-2 cells infected with HSV-1 KOS, 10 μM XZ45 inhibited viral DNA synthesis by 50-fold (Fig. 4B). The reduction in viral DNA synthesis appeared to be less than the 1,000-fold reduction in viral yields observed under these conditions. Therefore, we concluded that XZ45 inhibits HSV replication, at least in part, at the stages of viral DNA synthesis and late gene expression.

XZ45 inhibits HSV recombination in infected cells and in vitro. Because these compounds were originally designed as inhibitors of the HIV IN, a DDE recombinase (19), and because there is evidence that ICP8 is involved in recombination (13, 14), we hypothesized that the compounds might inhibit homologous recombination in HSV-infected cells. To test the effect of XZ45 on HSV recombination during viral replication, we coinfectend HEp-2
cells with two HSV mutant viruses, HSV-1 8LacZ (U2 gene lacZ fusion) and HSV-1 hr99 (U5 gene lacZ insertion) mutant viruses in the presence of increasing concentrations of either XZ45 or the viral DNA polymerase inhibitor phosphonoacetic acid (PAA), the latter as a control for inhibition of viral DNA replication, to inhibit viral replication to various extents. At 20 h after infection, the infected cultures were harvested, and progeny viruses were titrated on Vero cells (which exhibit plaques only with the wild-type [WT] recombinants) and V529 cells (which exhibit plaques only with the mutant viruses and the recombinants) to determine the viral titers of recombinant WT virus and total virus, respectively. When the recombination frequency was plotted relative to levels of viral replication, we observed that as XZ45 inhibited viral replication, the recombination frequency decreased significantly (P < 0.0117; Spearman’s rank correlation analysis, two-way analysis) (Fig. 5A). In contrast, inhibition of viral DNA replication with PAA did not significantly alter the frequency of recombinant viruses (P = 0.7966) (Fig. 5A). Therefore, XZ45 inhibits homologous recombination between viral genomes as well as inhibiting viral DNA synthesis.

The HSV ICP8 DNA-binding protein shows in vitro strand transfer activity, either alone (13) or in combination with a nuclease (14), and shows some properties of a DDE recombinase (11). We therefore tested if XZ45 could inhibit ICP8s in vitro recombinase activity in a strand invasion assay (13). In this assay we incubated ICP8 with an unlabeled double-stranded DNA molecule and a labeled single-stranded oligonucleotide, conditions under which ICP8 has been shown to promote formation of a D loop (13). We observed that XZ45 reduced the amount of the D loop formed (Fig. 5B). Therefore, XZ45 seemed to inhibit in vitro strand exchange by ICP8.

To test the hypothesis that XZ45 affects ICP8 function by inhibiting its binding to ssDNA, we first performed an electrophoretic mobility shift assay (EMSA) with purified ICP8 protein and a 32P-labeled ssDNA probe. XZ45 did not inhibit the ssDNA probe binding to ICP8, and there may have been a slight increase in DNA binding in the presence of XZ45 (Fig. 5A). In contrast, inhibition of viral DNA replication with PAA did not significantly alter the frequency of recombinant viruses (P = 0.7966) (Fig. 5A). Therefore, XZ45 inhibits homologous recombination between viral genomes as well as inhibiting viral DNA synthesis.

The HSV ICP8 DNA-binding protein shows in vitro strand transfer activity, either alone (13) or in combination with a nuclease (14), and shows some properties of a DDE recombinase (11). We therefore tested if XZ45 could inhibit ICP8s in vitro recombinase activity in a strand invasion assay (13). In this assay we incubated ICP8 with an unlabeled double-stranded DNA molecule and a labeled single-stranded oligonucleotide, conditions under which ICP8 has been shown to promote formation of a D loop (13). We observed that XZ45 reduced the amount of the D loop formed (Fig. 5B). Therefore, XZ45 seemed to inhibit in vitro strand exchange by ICP8.

To test the hypothesis that XZ45 affects ICP8 function by inhibiting its binding to ssDNA, we first performed an electrophoretic mobility shift assay (EMSA) with purified ICP8 protein and a 32P-labeled ssDNA probe. XZ45 did not inhibit the ssDNA probe binding to ICP8, and there may have been a slight increase in DNA binding in the presence of XZ45 (Fig. 5C). Second, in an ssDNA-coated-bead pulldown assay, we observed that XZ45 did not inhibit ICP8 binding to the beads when it was added either during both infection and pulldown or during the pulldown assay only (Fig. 5D, top). Consistent with this, XZ45 did not inhibit ICP8 binding to ssDNA-cellulose in a similar assay using HSV-1-infected cell lysates (Fig. 5D, bottom). In total, our results argue...
that XZ45 inhibits ICP8 recombinase activity without negatively affecting its ssDNA binding.

**XZ45 inhibits HCMV replication.** The other human herpesviruses also encode homologs of the ICP8 DNA-binding protein and the pUL15 terminase large subunit (20); therefore, they might also be sensitive to these compounds. The HCMV terminase enzyme is sensitive to raltegravir in vitro (10), but viral replication had not been tested. Therefore, we determined the effect of XZ45 on HCMV replication in human foreskin fibroblasts (HFFs). We incubated HFF cells with various concentrations of XZ45 from the time of infection with HCMV, harvested the cultures at 5 dpi, and titrated the viral yields. We observed that XZ45 inhibited HCMV replication very potently, with an EC_{50} of less than 50 nM and an efficacy of 10^4-fold reduction at 0.5 μM (Fig. 6).

**XZ45 inhibits KSHV reactivation from latent infection.** KSHV, a gammaherpesvirus, encodes a DNA-binding protein, ORF6, which shares sequence homology with the ICP8 protein (21), and a homolog of the pUL15 terminase protein (22), suggesting that this virus might also be sensitive to XZ45. Because KSHV establishes latency upon primary infection, we examined the effect of XZ45 on KSHV reactivation. Treatment of latently infected cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA/PMA) and the histone deacetylase (HDAC) inhibitor valproic acid (VA) induce reactivation of the virus (23). We reactivated 293 cells latently infected with a recombinant KSHV (rKSHV.219) with PMA/VA and cultured them in the presence of 10 μM XZ45 or dimethyl sulfoxide (DMSO) for 48 h. Treatment with the inhibitor did not cause major cytotoxicity, as more than 90% of the cells remained viable after treatment (results not shown). We observed a significant increase in KSHV early ORF57 and late ORF64 transcripts upon treatment with PMA and VA (Fig. 7). Treatment with 10 μM XZ45 significantly reduced these viral RNAs to levels near the uninduced levels (Fig. 7A and B). These results indicated that XZ45 inhibits KSHV reactivation from latency.

**DISCUSSION**

The most successful antivirals targeting the herpesviruses have been the nucleoside analogs (2). Under certain conditions, resistance to the nucleoside analogs can arise in HSV through accumulation of mutations in the viral thymidine kinase and/or DNA polymerase genes (2, 20). There have been limited second-line antivirals available for treatment of herpesvirus infections, but these have limitations, and although some new antiviral drugs are in various stages of development, there remains a need for new modalities, particularly for treatment of resistant infections (24). The herpesviruses encode two proteins that are known or predicted to contain an RNase H-like fold, which could be targeted by drugs and compounds similar to the drugs that target the HIV IN enzyme. A previous study had shown that raltegravir could inhibit the nuclease activity of the HCMV pUL89 protein (10), and we had shown that HSV-1 ICP8 had some molecular properties of a DDE recombinase (11). We therefore tested whether integrase inhibitors could inhibit replication of HSV and other herpesviruses and found that certain of these compounds were potent for inhibition of herpesviral replication. Thus, this class of enzymatic inhibitor has potential for antiviral therapy of herpesvirus infections as well as HIV infections. Our results could provide a scientific basis for the effect of raltegravir against recurrent HSV infection in a patient with NK cell deficiency (25).

**Mechanisms of inhibition of HSV replication.** The XZ45 compound reduces HSV-1 DNA replication, homologous recombination, and late gene expression. Thus, the earliest detectable effect of the compound is at the stage of viral DNA replication. Seven viral proteins are required for HSV DNA replication (26), including the ICP8 DNA-binding protein. We observed no effect of XZ45 on ICP8 DNA binding, but XZ45 did inhibit the strand transfer activity of ICP8. How the strand transfer activity of ICP8 might relate to DNA synthesis is unclear. HSV DNA synthesis has been proposed to occur through a two-stage mechanism in which the initial rounds of viral DNA synthesis are on a theta intermediate while later DNA synthesis occurs by a rolling circle mechanism (1). Some proposed mechanisms of HSV DNA replication involve strand transfer or recombinational mechanisms. For example, Roizman et al. (27) proposed that strand invasion mechanisms could be involved in the inversion of the L and S components of the genome, the regeneration of terminal sequences, and maintenance of the repeated sequences during viral DNA repli-
tion. Also, recombinational mechanisms have been proposed for the second stage of HSV DNA synthesis (28, 29). Therefore, the recombinase activity of ICP8 could be essential for viral DNA synthesis. Because XZ45 only partially inhibits viral DNA replication, it is interesting to speculate that the enzymatic activity that is inhibited involves the transition from theta form replication to rolling circle or the second stage of viral DNA synthesis. However, the reduction in viral DNA synthesis at 10 μM XZ45 is less than the reduction in viral yield, and XZ45 seems to be inhibiting late gene expression. ICP8 is known to promote late gene expression (30), and its recombinase activity may in some way exert a cis-acting effect on progeny viral DNA molecules that stimulates late gene transcription (31). XZ45 could inhibit this or other mechanisms stimulating late gene expression. Finally, an important part of the effect of XZ45 is likely to be exerted on the terminase nuclease activity (9, 10) and to inhibit cleavage and encapsidation of progeny viral DNA.

Inhibition of other herpesviruses. XZ45 also potently inhibits HCMV replication in human fibroblasts. Raltegravir had been shown to inhibit the HCMV terminase nuclease activity (10), and this may be at least a part of the target for XZ45 in HCMV-infected cells. The HCMV UL57 DNA-binding protein is highly homologous to the HSV-1 ICP8 DNA-binding protein, so this could also be a target for XZ45 in HCMV-infected cells.

XZ45 inhibited induction of the KSHV early ORF57 RNA during reactivation, so the target in KSHV-infected cells seems to be earlier than viral DNA synthesis, or the order of gene expression may differ from that in lytic infection. Alternatively, the target...
may have a role in the reactivation of the KSHV DNA from latent infection. It is conceivable that the KSHV ORF6 DNA-binding protein has a role in promoting the viral chromatin structure changes that are observed during reactivation from latent infection (32, 33).

Certain of the other herpesviruses, such as human herpesvirus 6 (HHV-6) and Marek’s disease herpesvirus, integrate into the host cell chromosome and persist in this novel form of latent infection (34–37). It is conceivable that the viral ICP8 homologs have a role in the integration or excision process through their recombinase activities and might be a target for this class of antiviral compounds.

Development of resistance. Further studies will be needed to determine if it is possible for the herpesviruses to mutate to resistance to these compounds and, if so, to identify the possible mechanisms of resistance to these inhibitors. We attempted to select for an HSV-1 variant that was resistant to XZ45, but passage of virus in increasing concentrations of XZ45 led to a virus that showed slightly increased replication relative to WT virus in the absence or the presence of XZ45 (S. M. Gregory, R. C. Colgrove, and D. M. Knipe, unpublished results). Illumina sequencing of the genome of the selected virus showed only three mutations in open reading frames, all in the ICP4 gene, which could explain the increased viral replication in general. Therefore, it may be difficult to select for resistance to this type of compound for any of several reasons, including (i) decreased viral fitness of the resistant virus, (ii) multiple viral targets, or (iii) a host target that is more important for viral replication than cell growth.

Structure-activity relationships of the compounds tested. We found that the hydrazide and oxoisoindole compounds were the most potent for inhibition of HSV-1 replication. The XZ45 hydrazide compound was chosen for further study because it showed good antiviral activity and limited cytotoxicity. Identification of the target molecule(s) will be needed to determine why the hydrazide compounds provide selectivity for viral replication. The different herpesviruses may also have different patterns of sensitivity, so each will need to be screened with this and other panels of compounds. The libraries of compounds from HIV integrase inhibitor screens could be sources of additional compounds that have anti-herpes activity.

This study forms the foundation for the development and study of a new class of herpesvirus family antivirals that could complement the nucleoside analogs, which target the viral DNA polymerase, or new antivirals that target the helicase-primase complex. Much needs to be done to follow up on these initial observations, including identification of the target(s), detailed analysis of structure-activity relationships, and definition of the mechanism of inhibition. There is a particular need for new anti-viral drugs for HCMV, HHV-6, and KSHV, which this approach might provide. HCMV seemed to be especially sensitive to our lead compound, so this could be a very profitable lead to develop further as a therapeutic for clinical application. The potential of developing drugs that inhibit both HIV and herpesvirus infection makes this class of compounds a very attractive approach for drug development. HSV provides a facile system for studying herpesviral lytic infection and the biochemical and molecular mechanisms

**FIG 6** Effect of XZ45 on HCMV replication. HCMV yield reduction assay with XZ45, performed in HFF cells as described for Fig. 1, except that progeny virus was harvested at 5 dpi.

**FIG 7** Effect of XZ45 on KSHV gene expression during reactivation. HEK293 cells latently infected with rKSHV.219 (293rKSHV.219) were reactivated with 20 ng/ml of PMA and 30 mM VA and treated with 10 μM of XZ45 or DMSO for 48 h. Total RNA was isolated, and the levels of ORF57 (A) and ORF64 (B) transcripts were measured by qRT-PCR. Viral transcript levels were normalized to cellular 18S rRNA levels. Bars represent means ± standard errors of the means (n = 3). One-way analysis of variance with repeated measures was followed by the Bonferroni posttest. **, P < 0.01.
by which the small molecules affect lytically infected cells, while the other herpesviruses provide systems for studying the establishment of and reactivation from latent infection and some infectious agents with great medical need for new strategies for intervention.

MATERIALS AND METHODS

Cells and viruses. HEP-2 cells, Vero cells, and human fetal foreskin fibroblasts (HFFs) were obtained from the American Type Culture Collection. Cells were propagated as described previously (11).

HSV-1 WT KOS virus (40) was grown and titrated on Vero cells. The low-passage-number HSV-1 strain F (41) and HSV-2 strain G (41) viruses were provided by Bernard Roizman (University of Chicago). The HSV-1 8lacZ virus (42) containing a lacZ insertion in the U5 gene was grown and titrated on Vero cells. Human cytomegalovirus strain AD169 was grown and titrated on HFF cells.

HSV growth assays were as described previously (11). Human CMV growth assays were as described previously (44). KHSV reactivation from latent infection in 293KHSV cells was conducted as described previously (23). Analysis of viral protein expression by Western blotting and analysis of viral DNA replication by real-time PCR were conducted as described previously (11).

HIV integrase inhibitors. Raltegravir and 118-D-24 were originally obtained from the NIAID Division of AIDS Reagent Repository. Additional compounds from the Zhao and Burke laboratories included diketo acids XZ119 and XZ230, oxoisoindolines XZ89, XZ90, XZ100, and XZ259, hydrazides XZ45, XZ15, XZ49, XZ48, and XZ50, and pyridinones XZ199, XZ319 and XZ320, oxoisoindoles XZ89, XZ90, XZ99, XZ100, and XZ259 (see Table S1 in the supplemental material for structures and references for these HIV-1 integrase inhibitors).

Purification of ICP8 and biochemical studies. ICP8 was purified as described previously from baculovirus vector-infected SF21 insect cells (11). EMSA was performed as described previously (11). A 25-mer oligo(dT) oligonucleotide [oligo(dT)$_{33}$] (Integrated DNA Technologies) was phosphorylated at its 5’ end with T4 polynucleotide kinase (NEB) using [y-32P]ATP (6,000 Ci/mmol, 10 mCi/ml; PerkinElmer), and unincorporated radioactive nucleotides were removed using an illustra MicroSpin G-50 column (GE Healthcare). Approximately 2 pmol of oligo(dT)$_{33}$ (based on the assumption of 100% recovery from the MicroSpin column) was incubated on ice with the indicated concentration of ICP8 with or without drug in ICP8 storage buffer for 30 min. The samples were resolved in a 5% native polyacrylamide gel, and the gel was dried and exposed to a phosphor storage screen for imaging. In vitro ICP8 DNA-binding assays were conducted as described previously (11, 45).

The DNA strand exchange assay reaction was performed as described previously (13) using ICP8 purified as described above, pUC18 plasmid, and 32P-labeled pUC-18 PB11 oligomer. After incubation, the reaction mixture was digested with proteinase K, and the products were resolved in a 1.2% agarose gel. The gel was dried and subjected to autoradiography.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01318-14/-/DCSupplemental.

Table S1, DOC file, 0.7 MB. Figure S1, TIF file, 8 MB.

ACKNOWLEDGMENTS

This research was supported by NIH grant AI063106 and a Feasibility Project Award from the Harvard University Center for AIDS research grant (F30 AI060354) to D.M.K.

We acknowledge the NIAID Division of AIDS Reagent Repository for raltegravir and 118-D-24. We thank Blair Strang for advice on the HCMV yield reduction assays.

REFERENCES


18. Zhang X, Pais GC, Svarovskaia ES, Marchand G, Johnson AA, Karki...