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Brief Article

# Peptide HIV-1 Integrase Inhibitors from HIV-1 Gene Products

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Anti-HIV peptides with inhibitory activity against HIV-1 integrase (IN) have been found in overlapping peptide libraries derived from HIV-1 gene products. In a strand transfer assay using IN, inhibitory active peptides with certain sequential motifs related to Vpr- and Env-derived peptides were found. The addition of an octa-arginyl group to the inhibitory peptides caused a remarkable inhibition of the strand transfer and 3′-end-processing reactions catalyzed by IN and significant inhibition against HIV replication.

## Introduction

Many antiretroviral drugs are currently available to treat human immunodeficiency virus type 1 (HIV-1) infection. Viral enzymes such as reverse transcriptase (RT[a]), protease and integrase (IN), gp41, and coreceptors are the main targets for antiretroviral drugs that are under development. Because of the emergence of viral strains with multidrug resistance (MDR), however, new anti-HIV-1 drugs operating with different inhibitory mechanisms are required. Following the success of raltegravir, IN has emerged as a prime target. IN is an essential enzyme for the stable infection of host cells because it catalyzes the insertion of viral DNA inside the preintegration complex (PIC) into the genome of host cells in two successive reactions, designated as strand transfer and 3′-end-processing. It is assumed that the enzymatic activities of IN have to be negatively regulated in the PIC during its transfer from the cytoplasm to the nucleus. Otherwise, premature activation of IN can lead to the autointegration into the viral DNA itself, resulting in an aborted infection. We speculate that the virus, rather than the host cells, must encode a mechanism to prevent autointegration. The PIC contains in association with the viral nucleic acid, viral proteins such as RT, IN, capsids ($p24^{CA}$ and $p7^{NC}$), matrix ($p17^{MA}$), p6 and Vpr, cellular proteins HMG I (Y), and the barrier to autointegration factor (BAF).[1−4] It is likely that, due to their spatial proximity in the PIC, these proteins physically and functionally interact with each other. For instance, it is already known that RT activity inhibited by Vpr,[5] and that RT and IN inhibit each other.[5−9] Vpr also inhibits IN through its C-terminal domain.[5,10] Because these studies suggest that PIC components regulate each other's function, we have attempted to obtain potent inhibitory lead compounds from a peptide fragment library derived from HIV-1 gene products, an approach which has been successful in finding a peptide IN inhibitor from LEDGF, a cellular IN binding protein.[11]

In this paper, we describe the screening of an overlapping peptide library derived from HIV-1 proteins, the identification of certain peptide motifs with inhibitory activity against HIV-1 IN, and the evaluation of effective inhibition of HIV-1 replication in cells using the identified peptide inhibitors possessing cell membrane permeability.

## Results and Discussion

An overlapping peptide library spanning HIV-1 SF2 *Gag*, *Pol*, *Vpr*, *Tat*, *Rev*, *Vpu*, *Env*, and *Nef*, provided by Dr. Iwamoto of the Institute of Medical Science at the University of Tokyo (Supporting Information, SI, Figure 2A), was screened with a strand transfer assay[12] in search of peptide pools with inhibitory activity against HIV-1 IN. The library consists of 658 peptide fragments derived from the HIV-1 gene products. Each peptide is composed of 10−17 amino acid residues with overlapping regions of 1−7 amino acid residues. Sixteen peptide pools containing between 16 and 65 peptides were used for the first screening at the final concentration of 5.0 $\mu$M for each peptide (SI Figure 2B ). This initial screening gave the results shown in Figure 1. Both Vpr and Env4 pools showed remarkable inhibition of IN strand transfer activity, and consequently a second screening was performed using the individual peptides contained in the Vpr and Env4 pools. A group of consecutive overlapping peptides in the Vpr pool (groups 13−15) and groups 4−6 and 20−21 in the Env4 pool were found to possess IN inhibitory activity (Figure 2). We focused on Vpr15 and Env4-4 peptides because they showed inhibitory activity against IN strand transfer reaction in a dose-dependent manner (Figure 3). The $IC_{50}$ values of Vpr15

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[a]Abbreviations: HIV, human immunodeficiency virus; IN, integrase; RT, reverse transcriptase; MDR, multidrug resistance; PIC, preintegration complex; BAF, barrier to autointegration factor; $R_8$, octa-arginyl.

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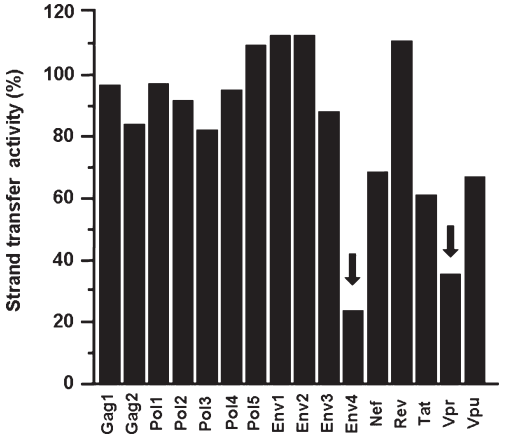


**Figure 1.** Inhibition of the IN strand transfer activity by peptide pools. Inhibition of the IN strand transfer activity was strongly inhibited by Env4 and Vpr pools (arrows). The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO).

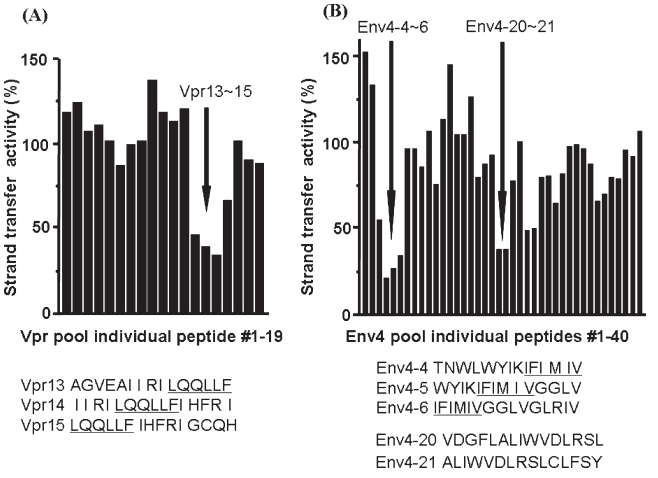


**Figure 2.** Identification of IN inhibitory peptides in the Vpr (A) and Env4 (B) pools based on the strand transfer activity of IN. The consecutive overlapping peptides display the inhibition of the strand transfer activity of IN (arrows). The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO). The concentration of each peptide was 5 $\mu$M. The common sequences of individual peptides derived from Vpr and Env4 pools with anti-IN activity are underlined.

and Env4-4 were estimated at 5.5 and 1.9 $\mu$M, respectively. These peptides did not show any significant inhibitory activity against HIV-1 RT, suggesting that they might inhibit IN strand transfer reaction selectively.

The overlapping peptides of Vpr13-15 and Env4-4-6 have the vommon hexapeptide sequences LQQLLF and IFIMIV, respectively. The LQQLLF sequence covers positions 64−69 of Vpr, which is a part of the second helix of Vpr. The IFIMIV sequence corresponds to positions 684−689 of gp160, which is a part of the transmembrane domain of TM/gp41. These hexapeptides are thought to be critical to inhibition of IN activity. It was recently reported[5] that similar peptides derived from Vpr inhibit IN with $IC_{50}$ values of 1−16 $\mu$M, which is consistent with our data. In this report,[5] the peptide motif was found to be 15 amino acid residues spanning LQQLLF from the overlapping Vpr peptide library. In our study, more precise mapping of inhibitory motif in Vpr peptides was achieved by identifying the shorter effective peptide motif. We focused on the Vpr-derived peptide, LQQLLF (Vpr-1) to develop potent inhibitory peptides. However, the expression of inhibitory activity against IN

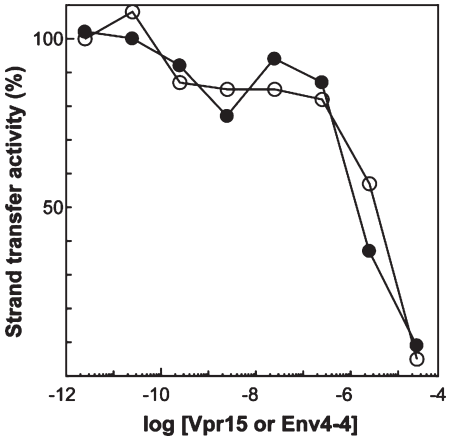


**Figure 3.** Concentration-dependent inhibition of IN strand transfer activities by Vpr15 (○) and Env4−4 (●) peptides. The *y*-axis represents the IN strand transfer activity relative to the solvent control (DMSO).

in vivo by only hexapeptides might be difficult because these hexapeptides penetrate the plasma membrane very poorly and to achieve antiviral activity, it is essential that they penetrate the cell membrane. To that effect, an octa-arginyl ($R_8$) group[13] was fused to the Vpr-derived peptides (Table 1). $R_8$ is a cell membrane permeable motif and its fusion with parent peptides successfully generates bioactive peptides without significant adverse effects or cytotoxicity.[14−18] In addition, the $R_8$-fusion could increase the solubility of Vpr-derived peptides which have a relatively hydrophobic character.

The inhibitory activity of Vpr-1 and Vpr-1-4 $R_8$ peptides against IN was evaluated based on the strand transfer and 3′-end-processing reactions in vitro (Table 1).[19,20] Vpr-1 did not show strong inhibition of either IN activity, but the $IC_{50}$ of Vpr-1 $R_8$ toward the strand transfer reaction of IN was 10-fold lower than that of Vpr-1 lacking the $R_8$ group. This indicates that the positive charges derived from the $R_8$ group might enhance the inhibitory activity of the Vpr-1 peptide. Because we were concerned that the strong positive charges close to the LQQLLF motif might interfere with the inhibitory activity, the 6 amino acid sequence (−IHFRIG−) was inserted as a spacer between LQQLLF and $R_8$ (Vpr-3 $R_8$). The IHFRIG sequence was used to reconstitute the natural Vpr. The $IC_{50}$ values of Vpr-2 $R_8$ for the strand transfer and 3′-end-processing activities of IN were 0.70 and 0.83 $\mu$M, respectively, while Vpr-3 $R_8$ showed potent IN inhibitory activities of 4.0 and 8.0 nM against the strand transfer and 3′-end- processing activities, respectively. This result indicates the additional importance of the IHFRIG sequence for inhibitory activities against IN. The increased IN inhibitory activities might be achieved presumably by the synergistic effect of the LQQLLF motif, the IHFRIG sequence, and the $R_8$ group. Vpr-4 $R_8$, in which the EAIIRI sequence was attached to further reconstitute the Vpr helix 2, showed inhibitory activities similar to those of Vpr-3 $R_8$, suggesting that reconstitution of helix 2 of Vpr is not necessary for efficient IN inhibition. Vpr-3 $R_8$ and Vpr-4 $R_8$, with $IC_{50} > 0.5\,\mu$M,[21] were less potent inhibitors of RT-associated RNase H activity, indicating that these peptides can selectively inhibit IN. These results suggest that Vpr-derived peptides are novel and distinct from any other IN inhibitors reported to date.

For rapid assessment of the antiviral effect of Vpr-derived peptides, we established an MT-4 Luc system in which MT-4 cells were stably transduced with the firefly luciferase expression cassette by a murine leukemia viral vector (SI Figure 3).

**Table 1.** Sequences of Vpr-Derived Peptides and Their $IC_{50}$ Values toward the Strand Transfer and 3′-End Processing Reactions of IN

| | sequence | $IC_{50}$ ($\mu$M) strand transfer | $IC_{50}$ ($\mu$M) 3′-end processing |
|---|---|---|---|
| Vpr-1 | LQQLLF | $68 \pm 1.0$ | $>100$ |
| Vpr-1 $R_8$ | Ac-LQQLLF -$R_8$-$NH_2$ | $6.1 \pm 1.1$ | $>11$ |
| Vpr-2 $R_8$ | Ac-IHFRIG-$R_8$-$NH_2$ | $0.70 \pm 0.06$ | $0.83 \pm 0.07$ |
| Vpr-3 $R_8$ | Ac-LQQLLF IHFRIG-$R_8$-$NH_2$ | $0.004 \pm 0.0001$ | $0.008 \pm 0.001$ |
| Vpr-4 $R_8$ | Ac-EAIIRI LQQLLF IHFRIG-$R_8$-$NH_2$ | $0.005 \pm 0.002$ | $0.006 \pm 0.006$ |

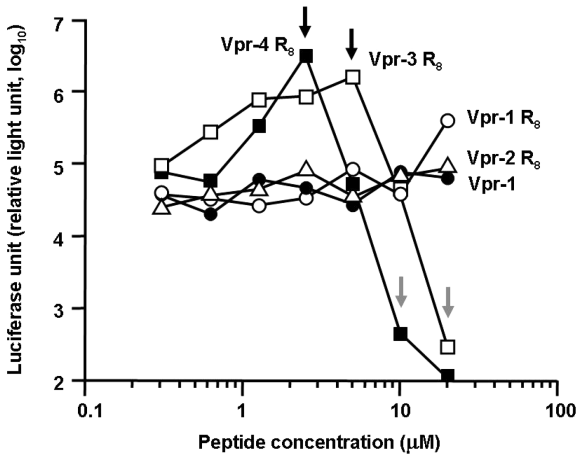


**Figure 4.** Luciferase signals in MT-4 Luc cells infected with HIV-1 in the presence of various concentrations of Vpr-derived peptides: Vpr-1 (●), Vpr-1 $R_8$ (○), Vpr-2 $R_8$ (△), Vpr-3 $R_8$ (□), Vpr-4 $R_8$ (■).

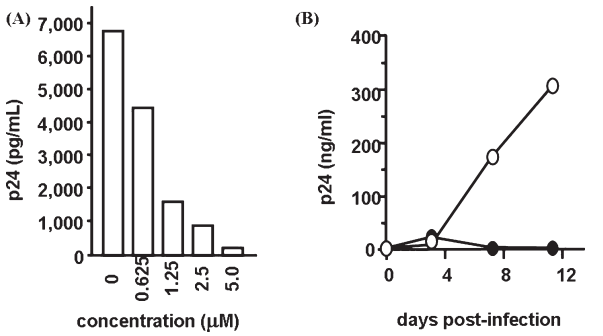


**Figure 5.** (A) The inhibition of HIV-$1_{JR\text{-}CSF}$ replication in NP2-CD4-CCR5 cells in the presence of various concentrations of Vpr-3 $R_8$. (B) The replication kinetics of HIV-$1_{HXB2}$ in MT-4 cells in the presence of Vpr-3 $R_8$ (●). The concentration of Vpr-3 $R_8$ was fixed at 0.5 $\mu$M. Absence of Vpr-3 $R_8$ (○).

MT-4 Luc cells constitutively express high levels of luciferase which are significantly reduced by HIV-1 infection due to their high susceptibility to cell death upon HIV-1 infection. Protection of MT-4 Luc cells from HIV-1-induced cell death maintains the luciferase signals at high levels. In addition, the cytotoxicity of Vpr-derived peptides can be evaluated by a decrease of luciferase signals in these MT-4 Luc systems. Vpr-2 $R_8$, which is a weak IN inhibitor, showed no significant anti-HIV-1 activity below concentrations of 20 $\mu$M, suggesting that its moderate $IC_{50}$ level in vitro is not sufficient to suppress HIV-1 replication in tissue culture and that the $R_8$ group is not significantly cytotoxic (Figure 4). Vpr-1 did not show any inhibitory effects against HIV-1 replication; however, Vpr-1 $R_8$ displayed a weak antiviral effect at a concentration of 20 $\mu$M and both Vpr-3 $R_8$ and Vpr-4 $R_8$ showed significant inhibitory effects against HIV-1 replication. The $R_8$ peptide did not show significant anti-HIV activity ($IC_{50} > 50$ $\mu$M, data not shown). These results suggest that the addition of the $R_8$ group enables Vpr-derived peptides to enter the cytoplasm and access IN, with the result that HIV-1 replication could be effectively inhibited.

Because Vpr-3 $R_8$ was less cytotoxic than Vpr-4 $R_8$, the inhibitory activities of Vpr-3 $R_8$ were further investigated. Two replication assay systems, R5-tropic HIV-$1_{JR\text{-}CSF}$ on NP2-CD4-CCR5 cells and X4-tropic HIV-$1_{HXB2}$ on MT-4 cells, were utilized. NP2-CD4-CCR5 cells were infected with HIV-$1_{JR\text{-}CSF}$ in the presence of various concentrations of Vpr-3 $R_8$. On day 4 postinfection, the culture supernatant was collected and the concentration of viral p24 antigen was measured by an ELISA assay. The p24 levels decreased in a dose-dependent manner with increasing the concentration of Vpr-3 $R_8$; 50% inhibition of p24 expression was obtained with approximately 0.8 $\mu$M of Vpr-3 $R_8$ (Figure 5A). This concentration was approximately 10-fold lower than the concentration of Vpr-3 $R_8$ known to be cytotoxic (Figure 4). Second, MT-4 cells were infected with HIV-$1_{HXB2}$ and the replication kinetics was monitored in the presence of 0.5 $\mu$M Vpr-3 $R_8$. The degree of replication of HIV-$1_{HXB2}$ was quite low in the presence of Vpr-3 $R_8$, while replication of HIV-$1_{HXB2}$ was robust in the absence of Vpr-3 $R_8$ (Figure 5B), suggesting that Vpr-3 $R_8$ strongly suppresses the replication of HIV-1 in cells. To examine whether the HIV-1 replication was blocked through the inhibition of IN activity, quantitative real-time PCR was performed. If IN is inhibited, the efficiency of viral genome integration should be decreased while the reverse transcription of viral genome should not be affected. Accordingly, NP2-CD4-CXCR4 cells were infected with HIV-$1_{HXB2}$ in the presence or absence of 0.5 $\mu$M Vpr-3 $R_8$. Genomic DNA was extracted on day 2 postinfection, and the viral DNA was quantified at the various steps of viral entry phase. The level of "strong stop DNA", representing the total genome of infected virus in Vpr-3 $R_8$-treated cells, was similar (139.7%) to that in DMSO-treated control cells and the level of viral DNA generated at the late stage of reverse transcription in Vpr-3 $R_8$-treated cells was slightly decreased (84.4%) compared to control cells. This small decline can probably be attributed to the weak anti-RNase H activity of Vpr-3 $R_8$. On the other hand, a drastic decrease of Alu-LTR products was observed in Vpr-3 $R_8$-treated cells (15.8%), indicating an inhibition of integrated viral genome. Concomitantly, the double LTR products, representing the end-joined viral genome catalyzed by host cellular enzymes, were increased by a factor of 8 (779.8%). These results strongly suggest that Vpr-3 $R_8$ blocks viral infection by inhibiting IN activity in cells, consistent with our in vitro observations. Judging by these results, Vpr-derived peptides with the $R_8$ group are potent IN inhibitors that suppress HIV-1 replication in vivo.

Finally, in silico molecular docking simulations of Vpr-derived peptides and HIV-1 IN were performed. The Vpr-derived peptides are located in the second helix of Vpr and were thus considered to have an α-helical conformation.[22] Docking simulations of three peptides (Vpr13, Vpr14, and Vpr15), using the predicted structure of the HIV-1 IN dimer as a template,[23] were performed by GOLD software to investigate the binding mode of the peptides, the binding affinity of

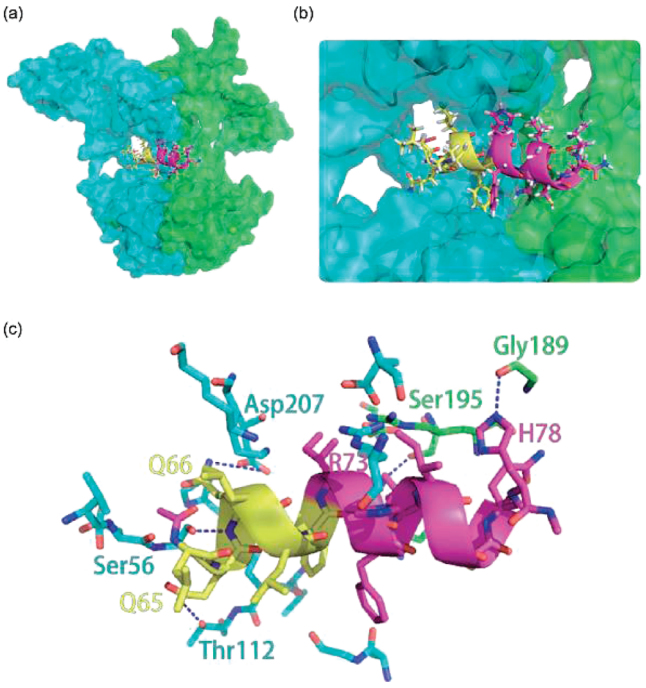


**Figure 6.** Predicted binding mode of Vpr15 to HIV-1 IN by GOLD. An overall view of (a) the complex obtained by docking Vpr15 with the HIV-1 IN dimer and (b) the closer view of the complex. The predicted structure of full-length HIV-1 IN was used as a template. Each HIV-1 IN monomer was shown as green or cyan surface. The docked Vpr15 is shown as a cartoon. The yellow-colored region is the LQQLLF motif. The GOLD score representing the docking complementarity is 69.83, indicating the high binding affinity between Vpr15 and IN. The hydrogen-bond interactions between HIV-1 IN and Vpr15 were presented by LIGPLOT software shown as blue dotted line (c).

the peptides being evaluated by GOLD Fitness score. The predicted binding mode of Vpr15 to IN is shown in Figure 6. Our results predict that the three Vpr-derived peptides interact with the cleft between the amino-terminal domain and the core domain of HIV-1 IN. This region is distinct from the nucleic acid interacting surfaces, indicating that the Vpr-derived peptides inhibit IN function in an allosteric manner. A previous report provided a model in which a Vpr peptide was bound to IN in a manner similar with our model[5] and, interestingly, the peptides were bound to IN with an exterior surface of Vpr. This earlier report that the full-length Vpr inhibits IN[10] strongly supports the predicted binding mode of Vpr15. Five hydrogen-bond interactions between HIV-1 IN and Vpr15 were identified by LIGPLOT analysis,[24] which invoked the following IN-Vpr amino acids: IN Thr112-Vpr Gln65, IN Ser56-Vpr Gln66, IN Asp207-Vpr Gln66, IN Ser195-Vpr Arg73, and IN Gly189-Vpr His78. The numbering of Vpr amino acids is based on the Vpr full-length coordinate, Figure 6. Additional hydrophobic contacts between IN and Vpr15 were found in which the following IN-Vpr amino acid pairs are involved: IN Lys211-Vpr Gln66, IN Pro109-Vpr Phe69, IN Arg262-Vpr His71, and IN Arg187-Vpr Gln77. These data indicate that the Gln65, Gln66, and Phe69 residues in Vpr-derived peptides play a major role in the interaction between IN and Vpr-derived peptides.

## Conclusions

In summary, two peptide motifs, LQQLLF from Vpr and IFIMIV from Env4, possessing inhibitory activity against HIV-1 IN, were identified through the screening of overlapping peptide library derived from HIV-1 gene products. We initially speculate that HIV encodes a mechanism to prevent autointegration in the PIC because integration activity must be regulated until the virus infects cells. This speculation is supported by the finding that IN inhibitors exist in the viral PIC components. Vpr-derived peptides with the $R_8$ group showed remarkable inhibitory activities against the strand transfer and 3′-end-processing reactions catalyzed by HIV-1 IN in vitro. In addition, Vpr-3 $R_8$ and Vpr-4 $R_8$ were shown to inhibit HIV-1 replication with submicromolar $IC_{50}$ values in cells using the MT-4 Luc cell system. In the quantitative analysis of p24 antigen, 50% inhibition of HIV-$1_{JR\text{-}CSF}$ replication was caused by approximately 0.8 $\mu$M of Vpr-3 $R_8$, and the replication of HIV-$1_{HXB2}$ was extensively suppressed in the long term by Vpr-3 $R_8$ at 0.5 $\mu$M concentrations. Our finding suggest that these peptides could serve as lead compounds for novel IN inhibitors. Amino acid residues critical to the interaction of Vpr-derived peptides with IN were identified by our in silico molecular docking simulations, and suggests that more potent peptides[25] or peptidomimetic IN inhibitors represent a novel avenue for future small molecule inhibitors of IN and HIV integration.

## Experimental Section

**Peptide Synthesis.** Vpr-derived peptides containing the $R_8$ group were synthesized by stepwise elongation techniques of Fmoc-protected amino acids on NovaSyn TGR resin. Coupling reactions were performed using 5.0 equiv of Fmoc-protected amino acid, 5.0 equiv of diisopropylcarbodiimide, and 5.0 equiv of 1-hydroxybenzotriazole monohydrate. Cleavage of peptides from resin and side chain deprotection were carried out with 10 mL of TFA in the presence of 0.25 mL of *m*-cresol, 0.75 mL of thioanisole, 0.75 mL of 1,2-ethanedithiol, and 0.1 mL of water as scavenger by stirring for 1.5 h. After filtration of the deprotected peptides, the filtrate was concentrated under reduced pressure, and crude peptides were precipitated in cooled diethylether. All crude peptides were purified by RP-HPLC and identified by MALDI-TOFMS. Purities of all final compounds were confirmed (>95% purity) by analytical HPLC. Detailed data are provided in SI.

**Enzyme Assays.** The strand transfer assay for the first screening was performed as described previously.[12] The IN strand transfer and 3′-end-processing assays for peptide motif characterizations were performed as described previously.[19,20] RNase H activity was measured as described by Beutler et al.[21]

**Replication Assays.** For HIV-1 replication assays, $1 \times 10^5$ cells were incubated at room temperature for 30 min with an HIV-1 containing culture supernatant (ca. 0.2−50 ng p24) and then washed and incubated. Culture supernatants were collected at different time points, and then the cells were passaged if necessary. Levels of p24 antigen were measured using a Retro TEK p24 antigen ELISA kit, according to the manufacture's protocol. Signals were detected using an ELx808 microplate photometer.

For MT-4 Luc assays, MT-4 Luc cells ($1 \times 10^3$ cells) grown in 96-well plates were infected with HIV-$1_{XHB2}$ (ca. 0.2−10 ng p24) in the presence of varying concentrations of Vpr-3 $R_8$. At 6−7 d postinfection, cells were lysed and luciferase activity was measured using the Steady-Glo assay kits according to the manufacture's protocol. Chemiluminescence was detected with a Veritas luminometer.

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**Supporting Information Available:** Additional experimental procedures including MS data and figures; HPLC charts of final compounds, explanation for HIV-1 genes and the peptide pools, and illustration of MT-4 Luc system. This material is available free of charge via the Internet at http://pubs.acs.org.

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