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Inhibition of Human Immunodeficiency Virus Type 1 Integration by Diketo Derivatives

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A series of diketo derivatives was found to inhibit human immunodeficiency virus type 1 (HIV-1) integrase activity. Only L-708,906 inhibited the replication of HIV-1(III_B) (50% effective concentration, 12 μM), HIV-1 clinical strains, HIV-1 strains resistant to reverse transcriptase or fusion inhibitors, HIV-2 (ROD strain) and simian immunodeficiency virus (MAC_{251}). The combinations of L-708,906 with zidovudine, nevirapine, or nelfinavir proved to be subsynergistic. In cell culture, addition of L-708,906 could be postponed for 7 h after infection, a moment coinciding with HIV integration. Inhibition of integration in cell culture was confirmed by quantitative Alu-PCR.

In an effort to improve combination therapy, to reduce viral replication even further, and to cope with virus strains that are resistant to multiple drugs, a search for inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase (IN) is ongoing. IN is responsible for inserting the viral cDNA into the host cell chromosome, an essential step in the replication of the virus (17, 24). Since no human counterpart of the enzyme is known, there is considerable interest in developing effective and selective inhibitors of the HIV integration process (8, 22).

The only viral enzyme required for HIV-1 integration is IN, a protein of 32 kDa encoded at the 3′ end of the *pol* gene (for a review, see reference 3). In the first step of the integration reaction, termed 3′-end processing, a dinucleotide (pGT) is removed from each 3′ end, to produce new 3′-hydroxyl ends (CA-3′-OH). This reaction occurs in the cytoplasm, within a large viral nucleoprotein complex, the preintegration complex (13). After entering the nucleus, the processed viral double-stranded DNA is joined to host target DNA. The joining reaction includes a coupled 5-bp staggered cleavage of the target host DNA and the ligation of processed CA-3′-OH viral DNA ends to the 5′-phosphate ends of the target DNA.

Although IN inhibitors can be evaluated in rather cumbersome preintegration complex assays (14), IN inhibition is typically assessed in oligonucleotide-based assays, using long terminal repeat (LTR) mimics to evaluate both processing and joining reactions in vitro (4, 25). Different classes of HIV-1 IN inhibitors have been reported (for a review, see reference 23). Most compounds, however, do not exhibit antiviral activity in cell culture. For those IN inhibitors that show an antiviral effect, it has not been unambiguously demonstrated that the integration step is targeted. On the contrary, both zintevir and L-chicoric acid, two compounds reported to target IN, were recently unmasked as inhibitors of viral adsorption (11, 21).

The diketo acids were reported to inhibit HIV-1 replication at micromolar concentrations through a specific inhibition of the DNA strand transfer step (10, 16). Selected resistant strains carried mutations in the IN gene. When these mutations were introduced into IN, they conferred partial resistance to the drugs. Recently, more-potent congeners of diketo acids that are capable of inhibiting HIV-1 replication in the nanomolar range have been synthesized (26). Another diketo derivative, 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)-propenone (5-CITEP), was cocrystallized with the catalytic core of HIV-1 IN (15). The compound bound to the active site and was reported to inhibit IN activity at micromolar concentration. No data on activity in cell culture have been reported. A derivative with antiviral activity, S-1360, is in clinical development (T. Yoshinaga, A. Sato, T. Fujishita, and T. Fujiwara, Abstr. 9th Conf. Retroviruses and Opportunistic Infections, p. 55, 2002). We have now evaluated the activities of the two originally published diketo derivatives together with newly synthesized analogues both in IN assays and in HIV-infected cells.

Inhibition of HIV-1 IN activity by diketo derivatives. All diketo derivatives were synthesized at the National Cancer Institute. Structures of the compounds are shown in Fig. 1. The structures of 5-CITEP (15) and L-708,906 (16) were published previously. The inhibition of 3′-end processing, DNA strand transfer, and overall integration by diketo derivatives was measured in standard oligonucleotide-based assays by using recombinant HIV-1 IN (7, 8). In the overall integration assay, inhibition of all three consecutive steps of the integration reaction, namely, binding of IN to DNA substrate, 3′-end processing, and DNA strand transfer, can be detected. A DNA strand transfer assay that measures integration of preformed complexes of IN and processed DNA into a target DNA molecule independently of other reaction steps had been previously designed (6). All molecules tested, apart from P4, were found to inhibit the 3′-end-processing reaction and the overall integra-

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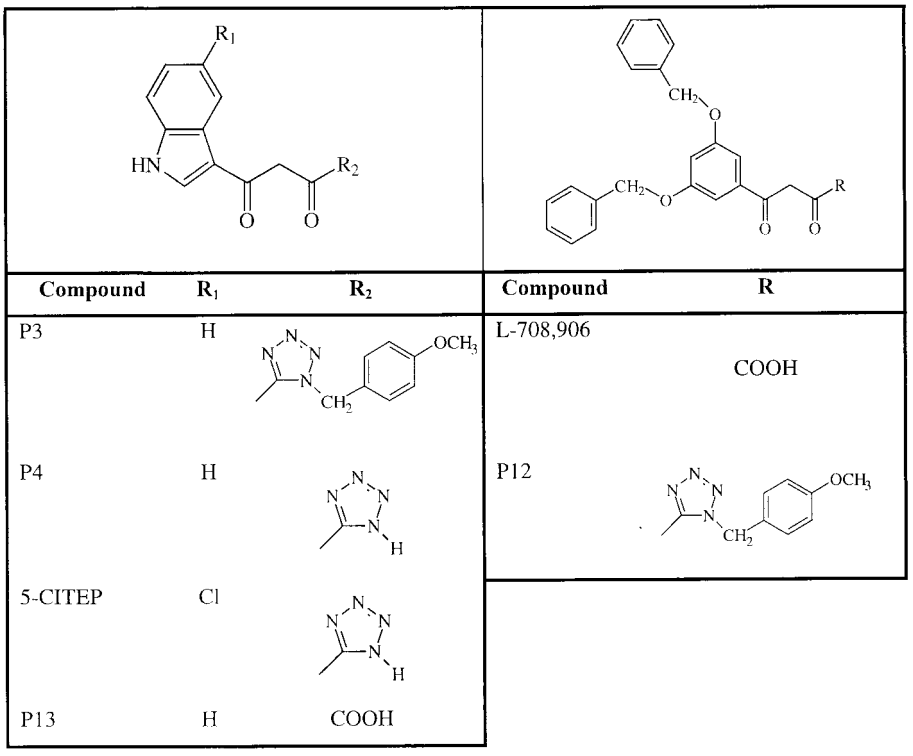

FIG. 1. Chemical structures of the diketo derivatives.

tion (Table 1). L-708,906 and P13 were the most potent congeners with 50% inhibitory concentrations (IC_{50}s) against overall integration of 0.2 and 1.1 μM, respectively (Table 1). Only two compounds were found to inhibit DNA strand transfer: L-708,906 was active at an IC_{50} of 3.5 μM, while P13 showed an IC_{50} of 15.1 μM. 5-CITEP was not found to be active in our DNA strand transfer assay.

Structure-activity relationship (SAR) for inhibition of HIV-1 IN activity. All compounds (Fig. 1) contain a central diketo moiety. 5-CITEP contains in addition an indole and a tetrazole group, whereas L-708,906 is composed of an additional 3,5-dibenzyloxyphenyl and a carboxyl group. P12 and P13 are chimeric molecules. When comparing P4 and 5-CITEP, it appears that the influence of the chloro substituent on the IN inhibition is almost negligible in all reactions. The *para*-methoxybenzyl group in P3 does not affect the inhibition of the overall integration reaction, when compared to P4. However, while P4 is inactive in the processing reaction, the *para*-methoxybenzyl substitution makes it more active in this reaction. Marked improvement in inhibition of IN activities was obtained by substitution of the tetrazole of P4 by a carboxyl functionality (compare the IC_{50}s of the overall integration reaction for P4 and P13). Substitution of the carboxyl group in L-708,906 by (*p*-methoxybenzyl)tetrazole reduced as in P12 the inhibition of overall integration 25-fold. The presence of a carboxyl group is required for potent inhibition of DNA strand transfer activity by L-708,906 and P13. When comparing the influence of the 3,5-dibenzyloxyphenyl moiety (L-708,906) with the indole moiety (P13), L-708,906 is fivefold more active in inhibiting overall integration than P13. L-708,906 is a more selective inhibitor of the DNA strand transfer reaction, whereas P13 is inhibiting 3′-end processing as well. When comparing P3 and P12, it appears that the 3,5-dibenzyloxyphenyl moiety increases the inhibitory potency in the overall integration assay (compared to the indole moiety).

TABLE 1. Inhibition of integrase activities by diketo derivatives

Compound	IC_{50} (μM)		
	3′ end processing	Overall integration	Strand transfer
P3	60.0 ± 11.7	127.3 ± 45.8	>533
P4	>392	101.1 ± 26.3	>784
5-CITEP	168.1 ± 33.1	44.2 ± 12.4	>690
L-708,906	20.5 ± 3.7	0.2 ± 0.07	3.5 ± 1.2
P12	21.7 ± 4.4	4.9 ± 0.7	>365
P13	6.5 ± 1.7	1.1 ± 0.7	15.1 ± 1.7

Inhibition of HIV and simian immunodeficiency virus (SIV) replication in cell culture by diketo derivatives. Next, we investigated whether the diketo derivatives were capable of inhibiting the replication of various strains of HIV-1, HIV-2, and SIV in MT-4 cells. Inhibition of viral replication was monitored by measuring viability of MT-4 cells 5 days after infection (20). Cytotoxicity of the compounds was determined in parallel by measuring the viability of mock-infected cells on day 5. The only compound with anti-HIV activity in cell culture was L-708,906 (data not shown). L-708,906 was active at a 50% effective concentration (EC_{50}) of 12.4 or 24.5 μM against HIV-1 strain III_B or HIV-1 strain NL4.3, respectively. The EC_{50} for inhibition of HIV-2 (ROD) was 62.1 μM, and for

TABLE 2. Antiviral activity against drug-resistant strains

Drug	EC_{50} (μM)[a]						
	Selected laboratory strain				Recombined (RVA) strain		
	WT strain (NL4.3)	NL4.3DS^{RES}	NL4.3AMD3100^{RES}	III_BNNRTI^{RES} (S0561945)	WT strain ($RAIII_BWT$)	NRTI^{RES} (PERE98287RT)	PI^{RES} (WOPRO1838)
Dextran sulfate	0.03 ± 0.01	**>8**	0.03 ± 0.01	—[b]	0.4 ± 0.2	0.4 ± 0.04	0.3 ± 0.1
AMD3100	0.02 ± 0.005	0.02	**>1.3**	—	0.03 ± 0.001	0.02	0.02
Nevirapine	0.06 ± 0.007	0.02	0.06	**>15**	0.05 ± 0.02	0.01 ± 0.001	0.05 ± 0.002
Zidovudine	0.005 ± 0.002	0.004 ± 0.001	0.006 ± 0.003	0.003 ± 0.001	0.005 ± 0.0006	**>8**	0.004 ± 0.001
L-708,906	24.5 ± 6.4	8.0 ± 1.7	8.4 ± 0.2	19.3 ± 14.7	10.1 ± 7.9	5.2 ± 1.2	7.4 ± 0.5
Ritonavir	0.08 ± 0.06	—	—	—	0.08 ± 0.03	0.1 ± 0.1	**1.6 ± 0.3**

[a] Strains selected in cell culture to be resistant to DS5000 (NL4.3DS^{RES}) (12) and to AMD31000 (NL4.3AMD3100^{RES}) (19) are tested in parallel with the parental strain (NL4.3WT). III_BNNRTI^{RES} is a selected strain resistant to NNRTIs. EC_{50} values for the parental III_B virus were identical to those for NL4.3. NRTI^{RES} (V. Arendt, Abstr. 4th Eur. Conf. Exp. AIDS Res., 1999) and PI^{RES} are recombinant strains carrying drug-resistant *RT* or *PRO* gene. $RAIII_BWT$ is the wild-type counterpart. Drug resistance is indicated in bold. WT, wild type.

[b] —, not determined.

inhibition of SIV (MAC_{251}) it was 22.6 μM. The cytotoxicity of the compounds was evaluated in parallel. All compounds proved relatively toxic in MT-4 cells. Due to the poor solubility of L-708,906, formation of crystals in the medium has been noticed at a concentration of ≥62 μM. Therefore, the real selectivity index of L-708,906 against the replication of HIV-1(III_B) in MT-4 cells could not be estimated at more than 5. L-708,906 was also active against the replication of HIV-1 in peripheral blood mononuclear cells (EC_{50}, 16.0 μM; 50% cytotoxic concentration [CC_{50}], 162.4 μM).

Inhibitory activity of L-708,906 against drug-resistant HIV-1 strains. To evaluate the potential clinical usefulness of an IN inhibitor, the activity of L-708,906 against drug-resistant HIV-1 strains was determined (Table 2). L-708,906 retained its activity when tested against an HIV-1 strain resistant to dextran sulfate, a virus adsorption inhibitor, and an HIV-1 strain resistant to the CXCR4 antagonist AMD3100. When inhibition of replication of an HIV-1 strain resistant to nucleoside reverse transcriptase inhibitors (PERE98287RT) (V. Arendt, Abstr. 4th Eur. Conf. Exp. AIDS Res., abstr. 71, 1999) or a strain resistant to nonnucleoside reverse transcriptase inhibitors (S0561945) was measured, the EC_{50}s obtained were comparable to those obtained with wild-type HIV-1 virus strains. No cross-resistance of L-708,906 was observed with an HIV strain resistant to protease inhibitors (WOPRO1838).

Combined inhibitory effects of L-708,906 and other HIV-1 inhibitors. The effect of combining diketo acids with other inhibitors in cell culture has not been reported. The combined inhibitory effect on HIV-1-induced cytopathic effect was examined by checkerboard combinations of various concentrations of the test compounds. The combined effect was analyzed by the isobologram method, as previously described (1). L-708,906 was added in combination with the nucleoside reverse transcriptase inhibitor zidovudine (3′-azido-3′-deoxythymidine [AZT]), the nonnucleoside reverse transcriptase inhibitor nevirapine, or the protease inhibitor nelfinavir to HIV-1-infected MT-4 cells. The combined inhibitory effects on HIV-1-induced cytopathic effect were evaluated by the isobologram method (Fig. 2). The $FIC_{nelfinavir} + FIC_{L\text{-}708,906}$ values fell between 0.5 and 1, implying a subsynergistic combinatory effect (Fig. 2). Likewise, the combinations of L-708,906 with zidovudine or nevirapine both also proved to be subsynergistic.

Time (site) of intervention. In the so-called time-of-addition experiment, one determines how long after infection the addition of an antiviral agent can be postponed without losing antiviral activity. Dextran sulfate, a polyanion, is known to interfere with the binding of the virus to the cell. The nucleoside analogue AZT inhibits the reverse transcription process, while ritonavir is an inhibitor of the proteolytic cleavage. Addition of these inhibitors can be delayed for 1, 4, and 18 to 19 h, respectively (Fig. 3). In this experimental setting, addition of L-708,906 could be postponed for 7 h, a time point believed to coincide with the event of integration. This compound can thus be utilized to pinpoint the integration step, and in particular the strand transfer step, in the synchronized HIV replication cycle.

Inhibition of lentiviral vector transduction. A single-round HIV transduction assay has recently been established by using HIV-1-derived vectors (2). In this assay, an epithelial cell line (e.g., 293T) is transduced with a replication-defective HIV-1 vector carrying a luciferase reporter gene (19). Since vectors are pseudotyped with VSV-G glycoprotein, HIV entry is not assayed for. In fact, transduction can be inhibited only selectively by inhibitors of reverse transcription and/or integration. L-708,906 was capable of inhibiting lentiviral transduction in this assay with an IC_{50} of 7.7 ± 0.7 μM, validating the potential to detect integration inhibitors by this assay. AZT and α-APA (loviride), known reverse transcriptase inhibitors, were able to inhibit lentiviral transduction as well (data not shown).

Analysis of HIV integration using real-time PCR. To verify that L-708,906 inhibits the integration step during HIV replication, DNA extracts of cells transduced with HIV vectors in the absence or presence of inhibitors were analyzed by quantitative PCR (Fig. 4). These tests measure the time course of the number of late reverse transcripts per cell (Fig. 4A), the number of 2-LTR circles (Fig. 4B) or the number of integrants (Fig. 4C), respectively. For each DNA species a specific TaqMan probe and primer set was designed (5). In the presence of 750 nM AZT, reverse transcription and 2-LTR circle formation are inhibited. As a result, no proviral DNA is detected by quantitative Alu-PCR. In the presence of L-708,906 a manifest increase in 2-LTR circles was observed (Fig. 4B) as well as a complete block in integration (Fig. 4C). There was only a marginal effect on reverse transcription. Therefore, L708,906

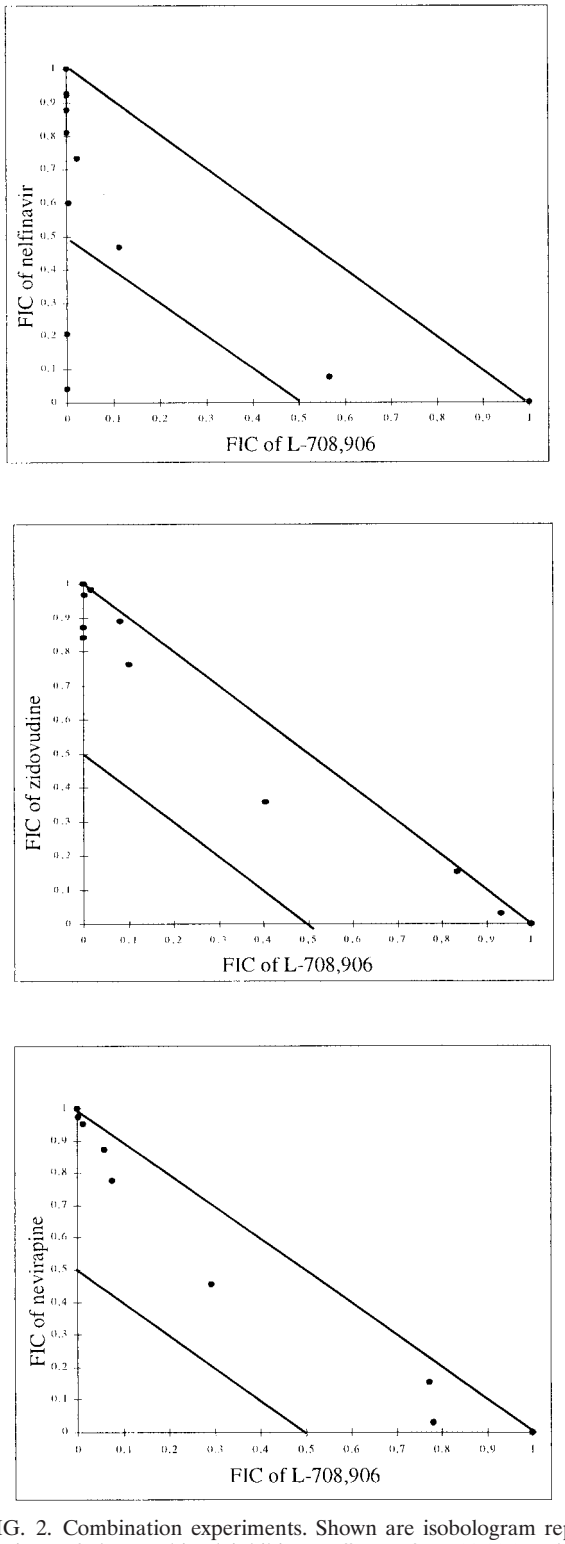

FIG. 2. Combination experiments. Shown are isobologram representations of the combined inhibitory effects of L-708,906 and the protease inhibitor nelfinavir (upper panel), the nucleoside reverse transcriptase inhibitor zidovudine (middle panel), and the nonnucleoside reverse transcriptase inhibitor nevirapine (lower panel) on the cytopathic effect of HIV-1(III_B) in MT-4 cells. Lines represent the unity lines for fractional inhibitory concentration (FIC) equal to 1 and 0.5, respectively.

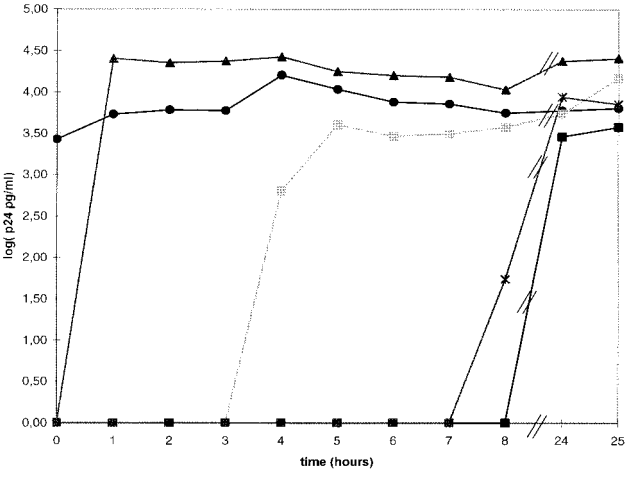

FIG. 3. Time-of-addition experiment. MT-4 cells were infected with HIV-1(III_B) at a multiplicity of infection of 0.5, and the test compounds were added at different times postinfection. Viral p24 Ag production was determined at 31 h postinfection and is expressed as the $\log_{10}$ of the p24 Ag content in picograms per milliliter. Symbols: ●, control; ▲, dextran sulfate (20 μM); ▨, AZT (1.9 μM); ■, ritonavir (2.8 μM); *, L-708,906 (173 μM).

can be considered an authentic IN inhibitor that owes its antiviral activity to inhibition of the integration process in HIV-infected cells.

Preclinical evaluation of diketo acids. Although the diketo acids are not the first HIV-1 IN inhibitors described that also display antiviral activity in cell culture, they are the first IN inhibitors suggested to inhibit HIV replication through a specific inhibition of the integration step (16). We have evaluated one of the active diketo acids reported, L-708,906, and compared its activity with the recently described 5-CITEP and several newly synthesized but structurally related molecules. Only L-708,906 displayed anti-HIV activity in cell culture. The EC_{50} obtained in our hands is higher than reported elsewhere (12.4 μM versus 2 μM). This may be explained by the differences in evaluation systems. Our assay for acute HIV infection is based on multiple replication rounds, whereas Hazuda et al. (16) used a single-round replication test. Moreover, because of the rather poor solubility of L-708,906, the variability in the data obtained was quite high. Clinically interesting features of in vitro drug performance were the following. The IN inhibitor was active against various HIV-1, HIV-2, and SIV strains, although with different levels of potency. No cross-resistance was observed with HIV strains resistant towards inhibitors of virus binding, fusion, or proteolytic processing. HIV strains resistant to nonnucleoside or nucleoside reverse transcriptase inhibitors were equally susceptible to inhibition by L708,906, as were wild-type strains. In combination experiments, L-708,906 proved to be subsynergistic with the reverse transcriptase inhibitors AZT and nevirapine and especially with the protease inhibitor nelfinavir, providing a rationale for combination therapy in patients.

We also compared the activity of L-708,906 with those of other diketo derivatives including 5-CITEP. Although all derivatives were active in the overall integration assay, only L-708,906 was active in cell culture. L-708,906 was more active

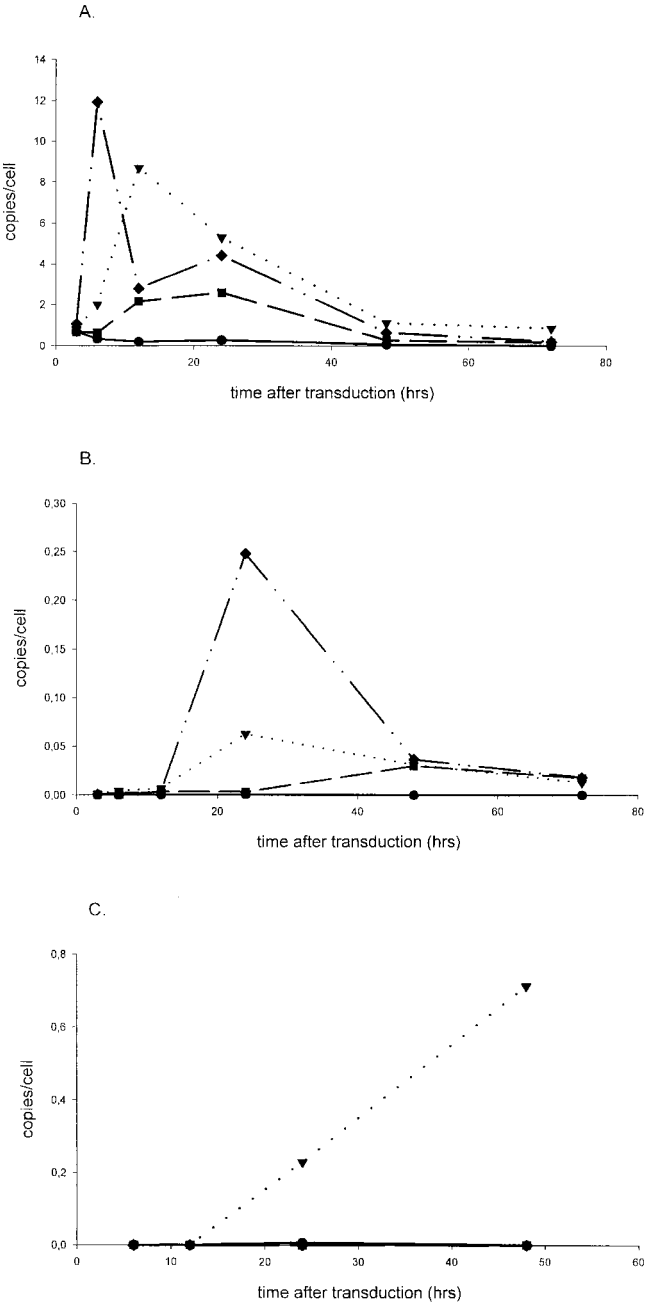

FIG. 4. Analysis of HIV integration by quantitative PCR. 293T cells were transduced with HIV-1 vectors at a multiplicity of infection of 10 in the absence (▼) or in the presence of 750 nM AZT (■) or 25 μM L-708,906 (♦). At different time points after infection, DNA extracts were prepared and analyzed by real-time PCR. Late reverse transcripts (A) were quantified, as well as 2-LTR circles (B) and integrated proviral DNA (C). A no-amplification control was run in parallel (●). The graph represents a typical experiment.

against strand transfer than against 3′-end processing. One new congener, P13, which is a hybrid molecule of L-708,906 with 5-CITEP, was identified as a potent inhibitor of HIV-1 IN. P13 inhibited both the 3′-end-processing step and the DNA strand transfer step. The differential inhibition of the strand transfer versus the 3′-end-processing reaction was also described for the related diketo acid analog L-731,988 (16). Based on our limited SAR analysis, it appears that inhibition of the DNA strand transfer required the presence of a carboxyl group. The potent inhibition of 3′ processing by 5-CITEP as opposed to L-708,906 was also noticed by Marchand et al. (18), who proposed the presence of two DKA binding sites, corresponding to the binding sites for viral DNA or target DNA, on the enzyme. Our analysis indicates that all derivatives of diketo acids should be tested for their ability to inhibit HIV-1 replication in cell culture. Although inhibitors that are more potent for IN inhibition than the original lead compounds may be found, this does not necessarily imply that the compounds are (more) active in cell culture. Unfortunately, the determinants for activity in cell culture are not those required for activity against the IN per se.

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