Among all the HIV-1 integrase inhibitors, the β-diketo acids (DKAs) represent a major lead in anti-HIV-1 integrase drug design. These derivatives inhibit the integration reaction in vitro with a strong specificity for the 3′-end joining step. They are also antiviral and inhibit integration in vivo. The aim of the present study has been to investigate the molecular interactions between DKAs and HIV-1 integrase. We have compared 5CITEP with one of the most potent DKAs reported by the Merck group (L-708,906) and found that 5CITEP inhibits 3′-processing at concentrations where L-708,906 is only active on strand transfer. We also report a novel bifunctional DKA derivative that inhibits 3′-processing even more effectively than 5CITEP. The interactions of these inhibitors with the viral DNA donor ends have been studied by performing experiments with oligonucleotides containing defined modifications. We propose that the bifunctional DKA derivative binds to both the acceptor and donor sites of HIV-1 integrase, whereas the monofunctional L-708,906 derivative binds selectively to the acceptor site.

Combination therapy including inhibitors of reverse transcriptase and protease has recently transformed the prognosis for AIDS. However, these treatments do not suppress viral replication in all patients, and the virus can remain active in some host tissues (cellular reservoirs) (1). It is therefore logical to look for agents that inhibit different viral targets, such as integrase.

Following viral entry and reverse transcription, HIV-1 viral DNA copies are integrated into host cell chromosomes. Integration is required for viral replication and is catalyzed by integrase, a viral enzyme encoded by the pol gene (Fig. 1 A) and generated after proteolysis of the gag-pol fusion protein precursor by the HIV-1 protease (for review, see Refs. 2–4). Fig. 1 summarizes the reaction catalyzed by HIV-1 integrase in vivo (panel A) and in vitro (panel B). In the cytoplasm, integrase catalyzes the removal of a GT dinucleotide immediately 3′ from a conserved CA dinucleotide at the 3′-end of both extremities of the viral genome (U3 and U5 long terminal repeats). After this first step, called 3′-processing, integrase remains bound to the long terminal repeats, and this preintegration complex (5–7) migrates to the nucleus where the second step of the integration reaction (3′-end joining or strand transfer) occurs. The 3′-end joining reaction consists of the direct nucleophilic attack of the 3′-recessed viral ends (donor DNA) on the host chromosome (acceptor DNA). Both termini of the viral DNA, which are kept in close proximity, integrate with a 5-bp stagger toward the 5′-ends of the target chromosomal DNA. Completion of the integration process requires removal of the two unpaired nucleotides at the 5′-ends of the viral DNA and gap-filling, probably accomplished by cellular enzymes.

The integration reaction can be reproduced and monitored in vitro using recombinant HIV-1 integrase and a radiolabeled DNA substrate (Fig. 1 B). In this assay, a 21-mer double-stranded DNA oligonucleotide corresponding to the 21 last bases of the U5 viral long terminal repeat, is used as substrate to follow both the 3′-processing and the strand transfer reactions. The 3′-processing reaction generates a 19-mer-labeled product, which can be integrated in another 21-mer double-stranded DNA oligonucleotide substrate during the strand transfer reaction. The integration can occur at different positions on the acceptor DNA molecule and leads to several reaction products migrating slower and faster than the original 21-mer substrate. We used the higher molecular weight species (Fig. 1 B, right panel) to quantify the strand transfer products (for review, see Ref. 8).

Many HIV-1 integrase inhibitors have been identified in the past few years using recombinant integrase (9–12), and recently a new family of antiviral inhibitors has been reported, the β-diketo acids (DKAs) (13). The DKAs such as L-708,906 (Fig. 2 A) exhibit potent activity against HIV-1 integrase in vitro and have the remarkable property of being selective for strand transfer. These compounds also reduce viral replication in cell culture. The validation of HIV-1 integrase as the target of DKAs was demonstrated by the selection of drug-resistant viruses bearing mutations in their integrase gene (13).

5CITEP is another inhibitor whose structure resembles that of DKAs (Fig. 2 A and Ref. 14). The carboxylic group of DKAs corresponds to the tetrazole of 5CITEP, which can be considered as an isosteric acid replacement. Interestingly, Davies and co-workers (14) reported the crystal structure of 5CITEP bound to the HIV-1 integrase active site in the vicinity of the enzyme catalytic residues Asp-64, Asp-116, and Glu-152.

The aim of the present study was to investigate the molecular interactions between DKAs and HIV-1 integrase. We have compared 5CITEP with one of the most potent DKAs...
reported by the Merck group and found that 5CITEP inhibits 3/11032-processing at concentrations where L-708,906 is only active on strand transfer. We also report a novel bifunctional DKA derivative that inhibits 3/11032-processing even more effectively than 5CITEP. The interactions of these inhibitors with the viral DNA donor ends have been studied by performing experiments with oligonucleotides containing defined modifications. We propose that the bifunctional DKA derivative binds to both the acceptor and donor sites of HIV-1 integrase, whereas the monofunctional L-708,906 derivative binds selectively to the acceptor site.

**MATERIALS AND METHODS**

DNA Oligonucleotides and Drugs—Oligonucleotides were purchased from IDT Inc. (Coralville, IA) and purified on a 20% (19:1) denaturing polyacrylamide gel using UV shadow. Purified oligonucleotides were

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5'-end-labeled by T4-polynucleotide kinase (Invitrogen) as described previously (8). The synthesis of DRAs and 5CITEP derivatives will be described in detail.2

HIV-1 Integrase Inhibition Assay—Unless otherwise indicated, the integrase-DNA complexes were preformed (16) by mixing 400 nM HIV-1 integrase with 5 nM 5'HQN-32P-labeled 21-mer double-stranded DNA template for 15 min on ice in a reaction buffer containing 25 mM MOPS, pH 7.2, 25 mM NaCl, 7.5 mM MmCl₂, 0.1 mg/ml bovine serum albumin, and 14.3 mM β-mercaptoethanol. Inhibitors were then added to the reactions in a final volume of 10 μl, and integration reactions were carried out for 1 h at 37 °C. Reactions were quenched by adding 10 μl of denaturing loading dye. Samples were loaded onto a 20% (19:1) denaturing polyacrylamide gel. Gels were exposed overnight and analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). The densitometric analysis was performed using ImageQuant from the Molecular Dynamics software package. Each lane was quantified to determine the amount of 3'-processing and strand transfer products, which were expressed as a fraction of the total radioactivity. Percentage of...

FIG. 2. Comparison of the inhibition of HIV-1 integrase by L-708,906 and 5CITEP. Panel A, structures of the two diketo acids tested. Panel B, PhosphorImager image of a typical experiment. Lane 17, DNA alone; lanes 1 and 15, DNA plus integrase. Concentrations of the diketo acids were: lanes 2 and 9, 0.1 μM; lanes 3 and 10, 0.4 μM; lanes 4 and 11, 1.2 μM; lanes 5 and 12, 3.7 μM; lanes 6 and 13, 11 μM; lanes 7 and 14, 33 μM, and lanes 8 and 15, 100 μM. Panel C, densitometric analysis of the gel shown in panel B. The graph represents the percentage of inhibition of the two integration steps as a function of the drug concentration. Inhibition of strand transfer is shown as circles; inhibition of 3'-processing is shown as triangles. Filled symbols, L-708,906; open symbols, 5CITEP. Panel D, schematic representation of the β-diketo acids.

FIG. 3. Comparison of the inhibition of HIV-1 integrase by 5CITEP and DKA1. A, structures of the two diketo acids tested. B, PhosphorImager image of a typical experiment. Lane 17, DNA alone; lanes 1 and 15, DNA plus integrase. Concentrations of the diketo acids were: lanes 2 and 9, 0.1 μM; lanes 3 and 10, 0.4 μM; lanes 4 and 11, 1.2 μM; lanes 5 and 12, 3.7 μM; lanes 6 and 13, 11 μM; lanes 7 and 14, 33 μM, and lanes 8 and 15, 100 μM. C, densitometric analysis of the gel shown in panel B. The graph represents the percentage of inhibition of the two integration steps as a function of the drug concentration. Circles, inhibition of strand transfer; triangles, inhibition of 3'-processing. Filled symbols, DKA1; open symbols, 5CITEP.
inhibition was computed using the integrase control (lane O in Figs. 2–5) as a reference.

Schiff Base Assay—The Schiff base assay was performed as described previously (8, 17). Briefly, uracil-containing oligonucleotides were 5’-end-labeled and annealed to their complementary strand. The resulting duplexes were then treated by 1 unit of uracil DNA glycosylase (Invitrogen) for 1 h at 37 °C to generate an abasic site. Drugs were incubated in a total volume of 10 μl with 1.5 μM HIV-1 integrase and 5 nM 5’-end-labeled abasic site-containing DNA template for 30 min at 37 °C in a buffer containing 25 mM MOPS, pH 7.2, 50 mM NaCl, 7.5 mM MnCl2, and 14.3 mM β-mercaptoethanol. Integrase-DNA cross-links were then reduced by treatment with 10 mM sodium borohydride. After 5 min at room temperature, samples were treated with one volume of 2× SDS-tricine loading buffer, heated 5 min at 95 °C, and loaded on 12–20% tricine-SDS-polyacrylamide gels (Invitrogen).

RESULTS

L-708,906 and 5CITEP Belong to the Same Family of β-Diketo Acids—We first compared the inhibition of HIV-1 integrase by one of the previously published DKAs, L-708,906 (13) and 5CITEP (Ref. 14 and Fig. 2A). Fig. 1B shows that both compounds inhibit the strand transfer step of the integration reaction and are markedly less effective on the 3’-processing step. Their IC50 values for strand transfer (concentrations that inhibit 50% of reaction) were comparable: 0.42 and 0.65 μM for L-708,906 and 5CITEP, respectively (Fig. 2A). However, their IC50 for 3’-processing differed significantly: >1000 and 35 μM for L-708,906 and 5CITEP, respectively. Thus, L-708,906 is more selective for strand transfer than 5CITEP by at least 40-fold (Fig. 2, panels B and C).

Fig. 2D shows a schematic representation of the general structure of DKAs, in which the R1 function is an acidic group and R2 an aromatic function. The two published compounds bear different acidic extremities (carboxylate versus tetrazole for L-708,906 and 5CITEP, respectively, Fig. 2A). To investigate the influence of this acidic function on HIV-1 integrase inhibition, we tested a 5CITEP analog in which the tetrazole group was replaced with a carboxylate (Fig. 3A). This new compound (DKA1) inhibited HIV-1 integrase similarly to 5CITEP (Fig. 3, B and C), indicating that the carboxyl and tetrazole functions are equivalent. Because DKA1 and L-708,906 are only different in their aromatic moiety (Fig. 2D), the difference observed for 3’-processing inhibition and selectivity for strand transfer for both compounds must be driven by this aromatic group.

A Bifunctional β-Diketo Acid Derivative Inhibits Both 3’-Processing and Strand Transfer—To elucidate the role of the aromatic portion of DKAs for strand transfer selectivity, we have designed derivatives of L-708,906 in which the aromatic moieties have been modified.2 Among them, the derivative with the most dramatic effect, DKA2, is presented in Fig. 4. In this molecule, the aromatic portion has been modified to bear a second diketo acidic side chain. This bifunctional DKA remained very effective on strand transfer (Fig. 4A) but inhibited 3’-processing with an IC50 below 10 μM, whereas L-708,906 was ineffective even at 1 mM (Fig. 4B). Furthermore, quantitation (Fig. 4C) revealed an accumulation of 3’-processing products for both compounds. The level of products decreased around 10 μM for DKA2 (IC50 for 3’-processing), whereas it reached a plateau above 1 μM for L708,906 (Fig. 4C). These data demonstrate that the aromatic portion of L-708,906 is responsible for strand transfer selectivity and that introducing a second diketo acid side chain renders the compound effective for 3’-processing.

Mono- and Bifunctional DKAs Inhibit HIV-1 Integrase Inde-
Independently of Their Order of Addition—Although all the previous results had been obtained by incubating the drug with preassembled enzyme-DNA complexes (16) (see “Materials and Methods”), we next investigated the activity of the DKAs when they were incubated first with the enzyme. For this purpose, we designed two protocols (Fig. 5A). In protocol 1, integrase-DNA complexes were formed in the absence of the drug for 15 min on ice in order to prevent catalysis. The drug was then added and the integration reaction initiated by placing the samples at 37°C for 1 h. In protocol 2, the drug was preincubated with HIV-1 integrase for 15 min at 37°C. Then the DNA was added, and reactions were continued for an additional hour. Fig. 5 (panels B and C) shows that both L-708,906 and DKA2 inhibit strand transfer similarly in protocols 1 and 2.

Preincubation with the enzyme (as in protocol 2) reduced the selectivity of L-708,906 for strand transfer by more than one order of magnitude (Fig. 5B). Under such conditions, L-708,906 inhibited 3’-processing almost totally at 1 mM (Fig. 5B, lane 15). Thus, our results demonstrate that both mono- and bifunctional diketo acids inhibit the strand transfer activity of HIV-1 integrase independently of the order of addition and that moderate 3’-processing inhibitory activity can be observed when the monofunctional DKA (L-708,906) was preincubated with integrase in the absence of DNA.

Differential Effects of Mono- and Bifunctional Diketo Acids on HIV-1 Integrase Binding to Donor DNA—To determine whether the inhibitory effect of the bifunctional compound DKA2 on 3’-processing was due to interactions with the donor DNA, we performed DNA-binding experiments using the Schiff base assay (17). In this assay (Fig. 6A), a DNA substrate containing a uracil is treated by uracil DNA glycosylase in order to generate an abasic site at a defined position on the oligonucleotide. When incubated with HIV-1 integrase, a Schiff base is generated between an appropriately positioned ε-amino group of a lysine present on the enzyme and the aldehydic abasic site. The DNA-protein complex is then covalently trapped (cross-linked) by reduction with sodium borohydride (17). In this set of experiments the same two preincubation conditions were examined (Fig. 5, protocols 1 and 2) using two different cross-linking positions (Fig. 6B, oligos 1 and 2). Using protocol 1, neither of the two DKAs inhibited DNA cross-linking (Fig. 6C). Similarly, L-708,906 had no effect on DNA cross-linking using protocol 2 (Fig. 6C). By contrast, using protocol 2, DKA2 inhibited HIV-1 integrase cross-linking with both oligonucleotides 1 and 2 (Fig. 6C). Our interpretation of the different results observed with DKA2 in the two protocols is that the Schiff base between integrase and its substrate is probably already formed during the preincubation on ice (protocol 1), prior to the addition of the drug. If DKA2 is added before the DNA, it forms a tight complex with the enzyme, which prevents binding of the DNA substrate. Our results demonstrate that the bifunctional molecule DKA2 can prevent the binding of the donor DNA, whereas the monofunctional L-708,906 does not.

DNA Structure Near the 3’-Processing Cleavage Site Influences the Inhibition of Strand Transfer by L-708,906—We next investigated the influence of the DNA structure at the extremity of the donor DNA. For this purpose, we used a pre-cleaved substrate (Fig. 7A) that mimics the 3’-processed substrate and allows monitoring of the strand transfer reaction without any interference from 3’-processing efficiency. To estimate the influence of the two unpaired nucleotides present on the non-
cleaved DNA strand after 3'-processing, we also used a pre-cleaved substrate in which the terminal base at the 5'-end on the non-cleaved strand had been replaced by an abasic site (Fig. 7A). The efficiency of inhibition of strand transfer by the monofunctional DKA L-708,906 was decreased by ~10-fold when using the pre-cleaved substrate and by ~30-fold when using the abasic site-containing pre-cleaved substrate (Fig. 7B and C). These results demonstrate the importance of the DNA structure near the 3'-processing cleavage site for the inhibition of strand transfer by L-708,906 and suggest an interaction between L-708,906 and the 3'-nucleophilic site of the donor DNA within the HIV-1 integrase-DNA complex.
DISCUSSION

To date, DKAs represent the most promising class of HIV-1 integrase inhibitors. In this study, we have demonstrated that the two previously published compounds L-708,906 (13) and 5CITEP (14) (Fig. 2A) belong to the same family, because the tetrazole group in the acidic extremity of 5CITEP can be replaced by a carboxylate. Additional evidence for this conclusion will be reported in a structure-activity study.2

We find that 5CITEP and L-708,906 exhibit a different selectivity for strand transfer, which may indicate a distinct mechanism of action (and enzyme binding) for both compounds. 5CITEP inhibits 3′-processing with an IC50 of 35 μM (Fig. 2A), whereas L-708,906 does not inhibit but rather enhances 3′-processing (Fig. 4C). This enhancement is probably a consequence of the selective strand transfer inhibition with accumulation of the 3′-processing product. The two compounds have the same central diketo function but have different aromatic and acidic extremities. The aromatic moiety cannot be held responsible for the selectivity difference because DKA1, which bears a carboxylate, exhibited the same potency as 5CITEP for 3′-processing. Therefore, the aromatic portion of the molecule determines the selectivity for strand transfer. This conclusion is further supported by the marked enhancement of the 3′-processing inhibitory activity exhibited by the bifunctional diketo acid DKA2, an analog of L-708,906 in which the aromatic portion has been reduced to a benzene ring bearing a second diketo chain (Fig. 4). The presence of an electronegative group on the DKA aromatic portion (Fig. 2D), i.e. a halogen or a carboxylate for 5CITEP and DKA2, respectively, appears to confer 3′-processing activity without affecting strand transfer inhibition.2 We have also found that DKA2 inhibited donor substrate binding (Fig. 6), demonstrating that this compound can compete with the donor DNA, whereas L-708,906 does not. The inhibition of 3′-processing and donor DNA binding by the bifunctional DKA2 but not by the monofunctional L-708,906 suggests that the second acidic function binds to the enzyme site that catalyzes 3′-processing. We refer to this site as the donor site.

We have found that the inhibitory activity of the monofunctional L-708,906 is decreased with substrates modified at their 3′-processing end. This was observed with a precleaved substrate and even further with an abasic site-containing precleaved substrate (Fig. 7, B and C). This suggests that L-708,906 binds near the nucleophilic end of the donor DNA, which would then inhibit strand transfer by interfering with and binding to an acceptor site where the enzyme catalyzes the nucleophilic attack of the 3′-OH end of the donor DNA toward the phosphodiester bond of the acceptor DNA.

We have proposed a distinct mode of binding for the monofunctional diketo acid derivatives. Because viral integrases catalyze the insertion of a donor DNA substrate into an acceptor DNA template, both DNA duplexes probably bind two adjacent sites. In our model (Fig. 8), monofunctional DKAs such as L-708,906 bind only to the acceptor site. Consequently, they do not inhibit 3′-processing and cannot compete for binding with the donor DNA (15). By contrast, the bifunctional DKA2 could bind both the donor and acceptor sites. At low concentration they would preferentially bind to the acceptor site, and at higher concentration they would bind to the donor site. Because no donor DNA was present in the co-crystal structure of 5CITEP-HIV-1 integrase obtained by Goldgur et al. (14), this structure may represent the drug bound to the donor site of the enzyme.

REFERENCES

1. Richman, D. D. (2001) Nature 410, 985–1001
2. Pommier, Y., Pilon, A. A., Bajaj, R., Mazumder, A., and Neamati, N. (1997) Antivir. Chem. Chemother. 8, 463–483
3. Brown, P. O. (1998) in Retroviruses (Coffin, J. M., Hughes, S. H., and Varmus, H. E., eds), pp. 161–203. Cold Spring Harbor Press, Cold Spring Harbor, NY
4. Skalka, A. M. (1999) in Retroviral Integration, Advances in Virus Research (Maramorosh, K., Murphy, F., and Shatkin, A. J., eds), p. 52. Academic Press, San Diego, CA
5. Bowerman, E., Brown, P. O., Bishop, J. M., and Varmus, H. E. (1989) Genes Dev. 3, 469–478
6. Farnet, C. M., and Haseltine, W. A. (1991) J. Virol. 65, 1910–1915
7. Miller, M. D., Farnet, C. M., and Bushman, F. D. (1997) J. Virol. 71, 3582–3590
8. Marchand, C., Neamati, N., and Pommier, Y. (2001) Methods Enzymol. 340, 624–631
9. De Clercq, E. (2000) Med. Res. Rev. 20, 323–349
10. d’Angelo, J., Mouscadet, J. F., Desmaele, D., Zouhiri, F., and Leh, H. (2001) Pathol. Biol. (Paris) 49, 237–246
11. Neamati, N., Marchand, C., and Pommier, Y. (2000) Adv. Pharmacol. 49, 147–165
12. Pommier, Y., Marchand, C., and Neamati, N. (2000) Antiviral Res. 47, 139–148
13. Hasuda, D. J., Pelock, P., Witmer, M., Wolfe, A., Stillmock, K., Grobler, J. A.,
14. Goldgur, Y., Craigie, R., Cohen, G. H., Fujiwara, T., Yoshinaga, T., Fujishita, T., Sugimoto, H., Endo, T., Murai, H., and Davies, D. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13040–13043

15. Espeseth, A. S., Felock, P., Wolfe, A., Witmer, M., Grobler, J., Anthony, N., Egbertson, M., Melamed, J. Y., Young, S., Hamill, T., Cole, J. L., and Hazuda, D. J. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11244–11249

16. Hazuda, D. J., Wolfe, A. L., Hastings, J. C., Robbins, H. L., Graham, P. L., LaFemina, R. L., and Emini, E. A. (1994) J. Biol. Chem. 269, 3999–4004

17. Mazumder, A., Neamati, N., Pilon, A. A., Sunder, S., and Pommier, Y. (1996) J. Biol. Chem. 271, 27330–27338