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Irreversible Inhibition of Human Immunodeficiency Virus Type 1 Integrase by Dicaffeoylquinic Acids†

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Human immunodeficiency virus type 1 (HIV-1) and other retroviruses require integration of a double-stranded DNA copy of the RNA genome into the host cell chromosome for productive infection. The viral enzyme, integrase, catalyzes the integration of retroviral DNA and represents an attractive target for developing antiretroviral agents. We identified several derivatives of dicaffeoylquinic acids (DCQAs) that inhibit HIV-1 replication in tissue culture and catalytic activities of HIV-1 integrase in vitro. The specific step at which DCQAs inhibit the integration in vitro and the mechanism of inhibition were examined in the present study. Titration experiments with different concentrations of HIV-1 integrase or DNA substrate found that the effect of DCQAs was exerted on the enzyme and not the DNA. In addition to HIV-1, DCQAs also inhibited the in vitro activities of MLV integrase and truncated variants of feline immunodeficiency virus integrase, suggesting that these compounds interacted with the central core domain of integrase. The inhibition on retroviral integrases was relatively specific, and DCQAs had no effect on several other DNA-modifying enzymes and phosphoryltransferases. Kinetic analysis and dialysis experiments showed that the inhibition of integrase by DCQAs was irreversible. The inhibition did not require the presence of a divalent cation and was unaffected by preassembling integrase onto viral DNA. The results suggest that the irreversible inhibition by DCQAs on integrase is directed toward conserved amino acid residues in the central core domain during catalysis.

An essential step in the life cycle of human immunodeficiency virus type 1 (HIV-1) and other retroviruses is integration of the double-stranded DNA copy of the retroviral genome into a chromosome of the host cell (19, 34, 51, 53). Integration requires DNA sequences at the ends of the linear viral DNA and a protein encoded by the viral *pol* gene, integrase (for reviews, see references 5 and 25). The process is initiated by an integrase-mediated endonucleolytic cleavage of two nucleotides from the 3′ end of each strand of linear viral DNA (3′-end processing). The newly created 3′-OH viral end is then joined by integrase to the cellular DNA (3′-end joining). The chemical mechanism for both 3′-end processing and 3′-end joining is a one-step in-line transesterification (18). In vitro, the 3′-end joining step is reversible, and the reverse reaction that resolves the intermediate into its viral and cellular DNA parts is called disintegration (10).

HIV-1 integrase, a 288-amino-acid peptide of 32 kDa, can be divided into three discrete domains, N terminus, core, and C terminus. Mutational analysis in vitro showed that the full-length integrase is required for carrying out 3′-end processing and 3′-end joining, though the core domain alone (amino acid residues 50 to 212) can mediate disintegration (7, 17, 57). The central core contains a DD35E motif, $DX_{39\text{-}58}DX_{35}E$, that is phytogenetically conserved among integrases of retroviruses and some transposons (30, 46). The conserved aspartic and glutamic acids may participate in the coordination of a divalent cation and may be involved in catalysis (32). Integrases containing a mutation in the DD35E domain are catalytically inactive (17, 35, 57).

Integrase is an appealing target for inhibitors that might be useful in treating retroviral infection because (i) integration is essential to the replication of retroviruses and (ii) integrase has no known functional analogue in human cells (for reviews, see references 20 and 47). Despite the critical role played by integrase in the retroviral life cycle, there is little information concerning chemical compounds that show selective inhibition against the viral enzyme. The major classes of integrase inhibitors that have been reported to date include aurintricarboxylic acid (14) and cosalane analogues (13), caffeic acid phenethyl ester (22, 23), DNA binders (4, 9, 22), topoisomerase inhibitors (8, 22), *bis*-catechols (33), nucleotides and oligonucleotides (36, 37, 41), tetracyclines (42), diarylsulfones (43), and arylamides (63). A majority of the compounds reported thus far are not selective for integrase. Aurintricarboxylic acid and related compounds also inhibit reverse transcriptase and other phosphoryltransferases (14). Inhibition of integrase by DNA binders and topoisomerase inhibitors is relatively weak and nonselective. Importantly, most of the information on integrase inhibitors is derived from in vitro experiments with purified integrase, and the protective effect of integrase inhibitors against HIV infection in tissue culture is either undetectable or has not been examined. Also, the mechanism of inhibition for most of the aforementioned compounds has not been determined.

We have described a novel class of integrase inhibitors, the dicaffeoylquinic acids (DCQAs; Fig. 1), that are isolated from medicinal plants from the Kallawaya of Bolivia (50). These compounds and a synthetic analogue, L-chicoric acid, are potent inhibitors of HIV-1 replication in cultured cell lines and catalytic activities of integrase in vitro. The IC_{50}s (concentration that exhibits 50% inhibition) for inhibiting HIV-1 replication in tissue culture and the catalytic activities in vitro range

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† This report is dedicated to the memory of Brian Reese and others who suffered from and died of AIDS.

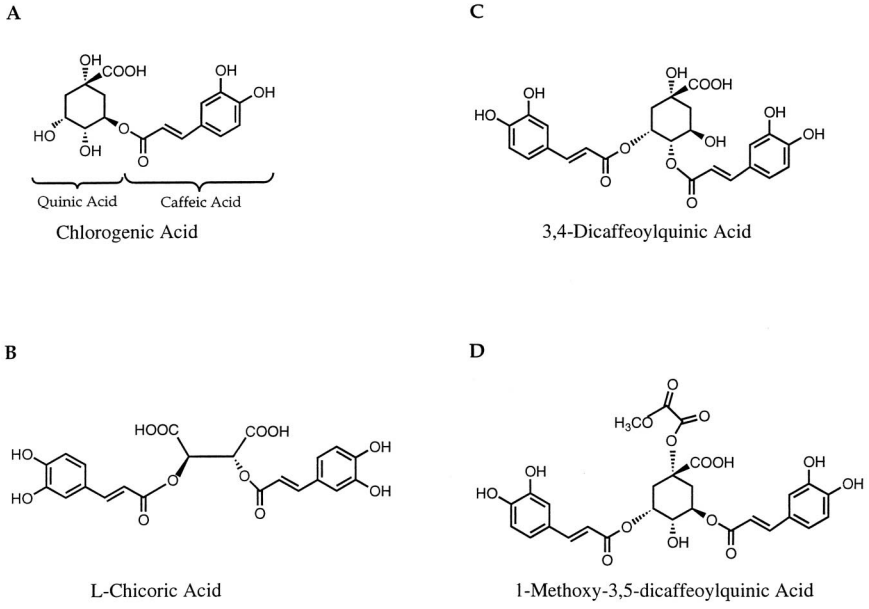

FIG. 1. Structures of DCQAs and related compounds. (A) Chlorogenic acid. The compound is composed of two moieties, quinic acid and caffeic acid, which are putative precursors for DCQAs. (B) L-chicoric acid. (C) 3,4-DCQA. (D) 1-MO-3,5-DCQA.

from 2 to 7 μM and 0.1 to 1 μM, respectively (49, 50). These compounds are relatively nontoxic and have LD_{50}s (dose that causes 50% cell death) in tissue culture at 700 μM or higher. The DCQAs have weak or no inhibition on gp120 binding to CD4, reverse transcriptase, and RNase H (39, 50). The likely precursors of DCQAs, chlorogenic acid, caffeic acid, and quinic acid, have no activity against HIV-1 in tissue culture or the purified integrase in vitro. An HIV-1 mutant containing a single glycine-to-serine substitution at position 140 of integrase displays resistance to L-chicoric acid, indicating that the compound acts at least in part against integrase in inhibiting viral replication in tissue culture (31). The DCQAs are therefore promising lead compounds for developing new antiretroviral drugs (42). In the present study, we examined the specific step at which DCQAs inhibit integration and the mechanism of inhibition of DCQAs on HIV-1 integrase.

MATERIALS AND METHODS

Enzymes and reagents. Recombinant integrases of HIV-1 and feline immunodeficiency virus (FIV) were purified as described previously (26, 52). Murine leukemia virus (MLV) integrase was a gift from P. O. Brown at Stanford University. T4 polynucleotide kinase, T4 DNA ligase, and restriction endonucleases were purchased from New England Biolabs; Ampli*Taq* DNA polymerase was from Perkin-Elmer Cetus; modified T7 DNA polymerase (Sequenase version 2.0) and exonuclease-free Klenow fragment of *Escherichia coli* DNA polymerase I were from U.S. Biochemicals. Deoxyribonucleotides were purchased from Pharmacia LKB. [γ-^{32}P]ATP was obtained from Amersham at a specific activity of 6,000 Ci/mmol. Oligonucleotides were purchased from Operon Technologies, Inc. Caffeic acid and chlorogenic acid were purchased from Aldrich Chemical Co., Inc.

DCQAs and analogues. Three compounds were examined for their mechanism of inhibition. The synthetic analogue L-chicoric acid (molecular weight = 474) was chosen because it has the most potent activity of all the structurally-related inhibitors tested (49). To ensure that the mechanism of action is generally applicable to the DCQA class of inhibitors, two additional compounds were selected: 1-methoxyoxalyl-3,5-dicaffeoylquinic acid (1-MO-3,5-DCQA; molecular weight = 602) and 3,4-DCQA (molecular weight = 516). L-chicoric acid was synthesized from the diphenylmethyl ester of L-tartaric acid by esterification with the bis-*O*-carboxymethylcaffeoyl chloride (50). 1-MO-3,5-DCQA was isolated from an aqueous extract of *Achyrocline satureioides* (50). 3,4-DCQA was synthesized as described previously (49).

Assays for integrase activity. The 3′-end processing, 3′-end joining, and disintegration activities of the fusion proteins were assayed as previously described (10, 58). The following oligonucleotides (Operon Technologies, Inc.) were used as DNA substrates: H-U5V1, 5′-ATGTGGAAAATCTCTAG**CA**GT (bold letters denote the invariant CA/TG dinucleotide pair); H-U5V1-2, 5′-ATGTGGAAAATCTCTAG**CA**; H-U5V2, 5′-AC**TG**CTAGAGATTTTCCACAT; H-V1/T2, 5′-ATGTGGAAAATCTCTAG**CA**GGCTGCAGGTCGAC; T1, 5′-CAGCAACGCAAGCTTG; T3, 5′-GTCGACCTGCAGCCCAAGCTTGCGTTGCTG. The oligonucleotides were purified by electrophoresis through a denaturing 15% polyacrylamide gel. Oligonucleotides H-U5V1, H-U5V1-2, and T1 were labeled at the 5′ end with [γ-^{32}P]ATP by using T4 polynucleotide kinase.

(i) 3′-end processing. Double-stranded oligonucleotides containing sequences derived from the U5 end of the HIV-1 long terminal repeat terminus was used as the DNA substrate to assay the 3′-end processing activity. The substrate was prepared by annealing the labeled H-U5V1 strand, which contains the conserved CA dinucleotide, with its complementary oligonucleotide H-U5V2. The 3′-end processing activity forms a labeled product that is shortened by two nucleotides.

(ii) 3′-end joining. To assay only the 3′-end joining activity independent of 3′-end processing, a substrate that resembles the viral U5 end after 3′-end processing was used. The preprocessed substrate was prepared by annealing the labeled H-U5V1-2 strand with the H-U5V2 strand. The joining activity is assayed by the appearance of labeled products that are longer than the input DNA on a denaturing gel. The lengths of the products are heterogeneous because the site of joining is largely random.

(iii) Disintegration. The disintegration substrate (Y oligomer) was prepared by annealing the labeled T1 strand with oligonucleotides T3, H-V1/T2, and H-U5V2 and purifying the annealed product on a native 15% polyacrylamide gel. The reaction is assayed by measuring the conversion of the labeled 16-nucleotide strand to a 30-nucleotide product.

(iv) Standard reaction condition. Typically, in a 20-μl volume, 0.1 pmol of the labeled DNA was incubated with 1.5 pmol of HIV-1 integrase for 60 min at 37°C in a reaction buffer containing a final concentration of 20 mM HEPES (pH 7.5), 10 mM $MnCl_2$, 30 mM NaCl, 10 mM dithiothreitol (DTT), 0.01 mM EDTA, 1 mM 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), and 0.05% Nonidet P-40.

In reactions that examined the effect of nucleoprotein complex formation on DCQA inhibition, a stable integrase-DNA complex was formed by preincubating the labeled DNA with integrase in the presence of $MnCl_2$ and reaction buffer for 20 min on ice or for 10 min at room temperature (16, 59). The DCQA was then added, and the sample was incubated at 37°C to start the reaction.

In all assays, the reaction was stopped by adding a final concentration of 18 mM EDTA, pH 8.0. The reaction products were mixed with an equal volume of loading buffer (98% deionized formamide, 10 mM EDTA [pH 8.0], 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 90°C for 3 min before analysis by electrophoresis on 15% polyacrylamide gels with 7 M urea in Tris-borate–EDTA buffer. Quantitation of the products was carried out with a Molecular Dynamics PhosphorImager.

Kinetic measurements. 3′-end processing activity of integrase was used as an index for measuring the effect of inhibitors on enzyme kinetics. HIV-1 integrase (75 nM) was incubated with the ^{32}P-labeled U5 DNA substrate (*s*) at a final

concentration of 50, 100, 200, or 400 nM in the presence or absence of 0.45 μM L-chicoric acid. Since integrase functions at least as a dimer (2), all of the substrate concentrations tested were in excess of the effective enzyme concentration. At various times after the start of the reaction, a 5-μl aliquot was removed for determining the extent of 3′-end processing. Reaction products were analyzed by electrophoresis on a 15% polyacrylamide gel with 7 M urea in Tris-borate–EDTA buffer. The amount of the product was determined by PhosphorImager analysis. The velocity (v, pM/min) of the reaction was calculated, and the K_m and V_{max} were determined by using a direct linear plot (s/v versus s) (28). The K_m and V_{max} values were expressed as mean ± standard error for three separate determinations. A two-tailed Student t test was used to identify differences between individual means. A P value of 0.01 or less was the criterion for statistical significance.

Dialysis experiments. HIV-1 integrase (75 nM) was preincubated with an inhibitor at its IC_{90} in a final volume of 150 μl of the standard reaction buffer for 20 min at 37°C. The sample was then transferred into a dialysis cassette (molecular weight cutoff, 10,000; Pierce) and dialyzed against 80 ml of reaction buffer at 4°C. After 4 h, the buffer was discarded, 80 ml of fresh reaction buffer was added, and the dialysis was continued for another 4 h. After dialysis, a 20-μl aliquot of the dialysate was removed, 1.5 pmol of the labeled U5 substrate was added to the dialysate, and 3′-end processing activity was assayed as described previously.

RESULTS

Inhibition of integration by DCQAs is selective and directed towards integrase and not DNA. Previous in vitro studies showed that DCQAs and a synthetic analogue, L-chicoric acid (Fig. 1, structures), inhibit all known catalytic activities of HIV-1 integrase (49, 50). It was not known, however, whether the inhibition by DCQAs was due to an interaction with integrase or the DNA substrate. To determine the target of action, we conducted integrase and DNA titration experiments (Fig. 2). In both experiments, the inhibitor L-chicoric acid was added at its IC_{50}. The IC_{50} for L-chicoric acid, determined by using 75 nM HIV-1 integrase and 5 nM U5 DNA substrate, was 150 nM (49, 50). In the integrase titration experiment, when the reaction was carried out at a fixed DNA concentration (5 nM) and at various concentrations of integrase, we found that the inhibition by L-chicoric acid could be overcome by the presence of high integrase concentrations (Fig. 2A). At integrase concentrations of 250 nM or higher, the product formation in the presence of L-chicoric acid was restored to about 83% of the inhibitor-free control. However, within the range of integrase concentration tested, the complete reverse of inhibition was not observed, and at present the reason is unclear. In the DNA titration experiment, when the reaction was carried out at a fixed integrase concentration (75 nM) and at various concentrations of DNA, the level of inhibition was not changed by increasing the concentration of the DNA substrate (Fig. 2B). Therefore, the titration experiments suggest that the inhibitor interacted with the enzyme and not the DNA substrate. Furthermore, the lack of an effect with an increase in DNA concentration suggests that the compound was not acting as a competitive inhibitor.

DCQAs are relatively nontoxic in cell culture (LD_{50}s ≥ 700 μM) and have weak or no inhibition on gp120 binding to CD4 or on HIV-1 reverse transcriptase or RNase H activities (39, 50). To further examine the specificity of inhibition, we determined the effect of L-chicoric acid and 1-MO-3,5-DCQA on several DNA-modifying enzymes and phosphoryltransferases, including the restriction enzyme *Hin*dIII, T4 DNA ligase, T4 polynucleotide kinase, and T7 DNA polymerase. The activities of these enzymes were assayed under conditions recommended by the manufacturers. Neither L-chicoric acid nor 1-MO-3,5-DCQA showed any effects on these enzymes at a concentration of 150 μM (data not shown). This result further demonstrates that DCQAs are selective inhibitors of integrase.

Activity of DCQAs against the catalytic core domain is not unique to HIV-1 integrase. In a previous study with truncated

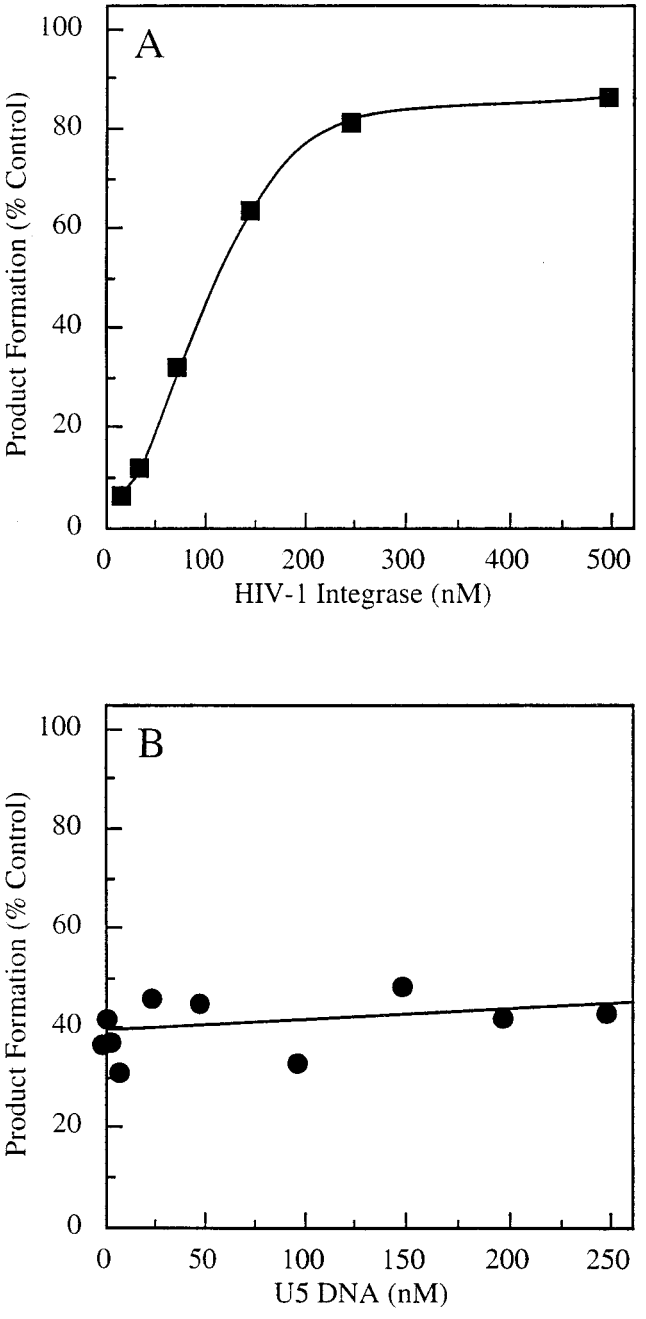

FIG. 2. Inhibition of 3′-end processing by L-chicoric acid depends on enzyme concentration and is independent of DNA concentration. (A) Integrase titration. The 3′-end processing reaction was carried out at 37°C for 30 min in the presence of 5 nM U5 DNA substrate (H-U5V1/H-U5V2), 150 nM (IC_{50}) L-chicoric acid, and various concentrations (15 to 500 nM) of HIV-1 integrase. (B) DNA titration. The reaction procedure was identical to that shown in panel A, except that the integrase concentration was fixed at 75 nM and the U5 DNA concentration was varied from 1 to 250 nM. In both panels A and B, the formation of the 3′-end processing product in the presence of L-chicoric acid is presented as percent of control; the control for each value represents a reaction with identical DNA and integrase concentrations but without L-chicoric acid. In panel A, the percents of DNA substrate converted to the 3′-end processing product in the control reaction by using 18.8, 37.5, 75, 150, 250, and 500 nM integrase were 3.5, 9.0, 18.1, 26.7, 40.3, and 48.7, respectively. In panel B, the percents of product formation of the control at 1.25, 2.5, 5, 10, 25, 50, 100, 150, 200, and 250 nM of U5 DNA were 20.2, 18.6, 16.8, 12.2, 10.4, 6.0, 2.8, 1.8, 1.4, and 0.8, respectively.

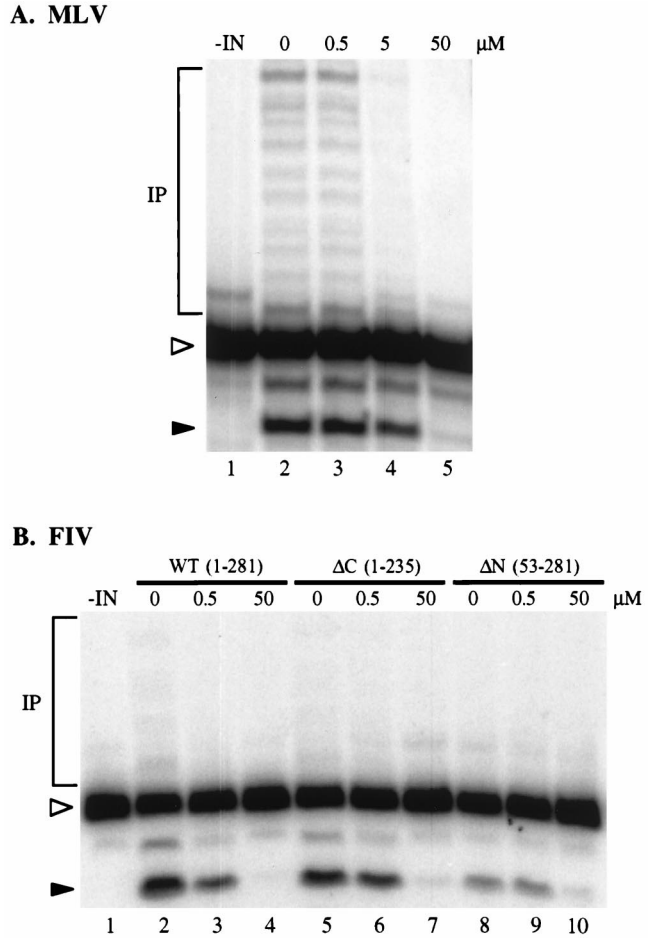

FIG. 3. Inhibition of MLV and FIV integrases by L-chicoric acid. (A) MLV integrase. The reaction was carried out at 37°C for 60 min under conditions as described previously (15). The reaction contained 50 nM MLV integrase, 5 nM U5 DNA substrate, and L-chicoric acid. Lane 1 shows a reaction without integrase. Lane 2 shows a reaction done in the absence of inhibitors; an equal volume of water was added instead. The numbers above the figure indicate the concentration of L-chicoric in the reaction. (B) FIV integrase. The reaction was carried out at 37°C for 60 min with 5 nM FIV U5 substrate (52) and the indicated concentrations of L-chicoric acid in the absence (lane 1) or presence of 75 nM wild-type (WT; lanes 2 to 4), C-terminal truncated (ΔC; lanes 5 to 7), or N-terminal truncated variants (ΔN; lanes 8 to 10) of FIV integrase. Numbers in parentheses represent the amino acid residues of the protein. The full-length and the truncated derivatives of FIV integrase contained a poly-His tag at the N terminus. The presence of the tag restores the 3′-end processing and joining activities of the truncated variants (52). In both panels A and B, the open and filled arrowheads indicate the positions of the labeled substrate and the processed product, respectively. The 3′-end joining products are indicated by IP.

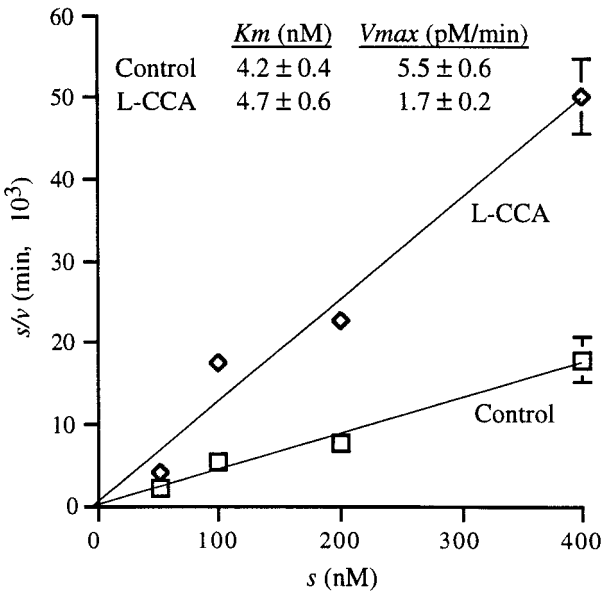

FIG. 4. Kinetic analysis of integrase inhibition by DCQAs. 3′-End processing reactions were carried out with 75 nM HIV-1 integrase and 50, 100, 200, or 400 nM U5 DNA substrate (*s*) in the absence (□) or presence (◇) of 450 nM L-chicoric acid. Aliquots of the reaction mixture were removed at various times after the start of the reaction, the yield of the 3′-end processing product was determined, and the initial velocity (*v*) at each substrate concentration was calculated. The time points used for rate determination at various substrate concentrations were as follows: for 50 nM, 5, 10, 15 min; for 100 nM, 10, 20, 30 min; for 200 and 400 nM, 20, 40, 60 min. The values of *v*/*s* were then plotted against *s* to determine the K_m and V_{max} (direct linear plot) (28). The K_m (nanomolar) and V_{max} (picomolar/minute) values were expressed as means ± standard errors for three separate determinations.

proteins, the action of DCQAs was mapped to the core domain of HIV-1 integrase (49). Since the core domain contains the active site and amino acid residues that are phytogenetically conserved, we tested whether DCQAs could inhibit integrases isolated from other species, MLV and FIV. The amino acid sequence of HIV-1 integrase is 21 and 37% identical to that of MLV and FIV integrases, respectively (1, 54, 55). We found that the synthetic analogue, L-chicoric acid, inhibited the catalytic activities of MLV (Fig. 3A) and FIV integrases (Fig. 3B, lanes 1 to 4) in a dose-dependent manner. However, the IC_{50}s of L-chicoric acid for MLV and FIV integrases (3.25 and 0.22 μM, respectively) were higher than that for HIV-1 integrase (0.15 μM). L-chicoric acid also inhibited 3′-end processing and joining mediated by the N-terminal truncated (deletion of amino acid residues 1 to 52) and C-terminal truncated (deletion of amino acid residues 236 to 281) variants of FIV integrase (Fig. 3B, lanes 5 to 10). These results suggest that DCQA inhibition was targeted towards the central core domain of integrase. The inhibition is not unique to HIV-1 integrase and may include the retroviral integrase subfamily.

DCQAs irreversibly inhibit HIV-1 integrase. The results in Fig. 2 suggest that DCQAs are not competitive inhibitors. To deduce the mode of inhibition, the 3′-end processing activity was used for kinetic analysis. The initial velocity (*v*) of the 3′-end processing reaction at different substrate concentrations (*s*) was determined in the presence or absence of an inhibitor. The data collected were used to calculate the K_m and V_{max} by using a direct linear plot (*s*/*v* against *s*) (Fig. 4). The differences in K_m and V_{max} in the absence or presence of an inhibitor were then used to determine the mode of inhibition (Fig. 4). The addition of L-chicoric acid lowered the V_{max} and did not change the K_m of the 3′-end processing reaction (Fig. 4), indicating that the compound affected integrase by noncompetitive or irreversible inhibition (28). Addition of 3,4-DCQA or 1-MO-3,5-DCQA also lowered the V_{max} and did not change the K_m (12a).

To confirm that the inhibition by DCQAs was irreversible, we carried out a dialysis experiment (Fig. 5). Integrase was preincubated in the presence of L-chicoric acid at its IC_{90} concentration. After preincubation, the inhibitor was removed by dialyzing the sample against the reaction buffer. After dialysis, the integrase was tested for its ability to mediate 3′-end processing and joining. Dialysis cassettes containing only the integrase or the inhibitor were used as positive controls. As expected, when the dialysis cassette contained only integrase,

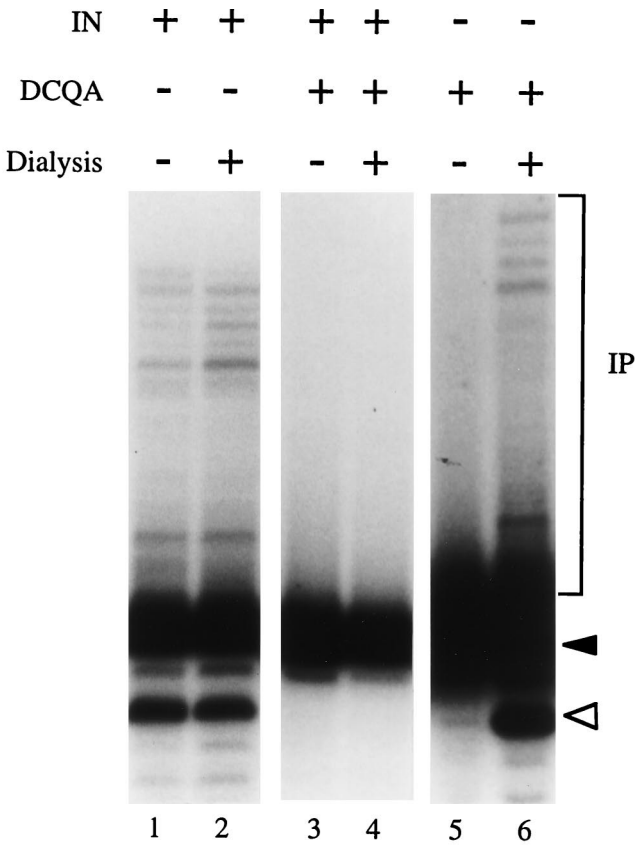

FIG. 5. Inactivation of integrase by L-chicoric acid cannot be reversed by dialysis. HIV-1 integrase (75 nM) was preincubated with 1 μM (IC_{90}) L-chicoric acid in 150 μl of the reaction buffer (20 mM HEPES [pH 7.5], 10 mM $MnCl_2$, 30 mM NaCl, 10 mM DTT, 0.01 mM EDTA, 1 mM CHAPS, and 0.05% Nonidet P-40) at 37°C for 30 min. The sample was then transferred into a dialysis cassette and dialyzed against 2× 80-ml reaction buffer at 4°C for 8 h. After dialysis, a 20-μl aliquot of the dialysate was removed, 5 nM of the U5 DNA substrate was added, and the reaction was allowed to proceed at 37°C for 30 min (lane 4). Lane 3 shows a reaction identical to that of lane 4, except that the sample was not subject to dialysis. In lanes 1 and 2, integrase was preincubated in the absence of L-chicoric acid, and the sample was (lane 2) or was not (lane 1) dialyzed before the addition of U5 DNA. In lanes 5 and 6, 1 μM L-chicoric acid was preincubated in the absence of integrase, and the sample was dialyzed (lane 6) or not dialyzed (lane 5) before the reaction was started by adding U5 DNA and 75 nM integrase. The symbols have the same meanings as those in the legend for Fig. 3.

subsequent additions of the DNA substrate showed a normal reaction (Fig. 5, lane 2). When integrase was preincubated with the inhibitor and then subjected to dialysis, the protein remained inactive even after dialysis (Fig. 5, lane 4). Similar results were obtained when the reaction was carried out by using 3,4-DCQA or 1-MO-3,5-DCQA as the inhibitor (data not shown). Since the inhibitors were adequately removed during dialysis (Fig. 5, lane 5 versus lane 6 and data not shown), the persistence of inactivity after dialysis confirmed that DCQAs acted as irreversible inhibitors.

Interaction of integrase with DCQAs does not require a divalent cation. The activity of integrase has an absolute requirement for a divalent cation, Mg^{2+} or Mn^{2+}. A divalent cation concentration of 1 mM or less does not support catalytic activities (10a). Under the standard reaction condition, the DCQAs are negatively charged. Since the inhibition by DCQAs is unchanged in the presence of 5, 10, or 15 mM Mn^{2+} (10a), it is unlikely that the mechanism of DCQA inhibition is by cation chelation. To confirm that inhibition of integrase by DCQAs did not require a divalent cation, HIV-1 integrase was preincubated with DCQAs in the presence or absence of Mn^{2+}. After preincubation, the divalent cation and the inhibitor were removed from integrase by dialysis. After dialysis, U5 DNA substrate and $MnCl_2$ were added, and the activity of integrase was determined by the 3′-end processing and joining reactions. Preincubation of integrase with L-chicoric acid (Fig. 6) or 1-MO-3,5-DCQA (data not shown) resulted in a complete absence of 3′-end processing and joining activities, regardless of whether the preincubation was done in the absence or presence of Mn^{2+} (Fig. 6, lanes 2 to 3 versus lanes 5 to 6). A normal reaction was observed when fresh integrase was added together with U5 DNA substrate and $MnCl_2$ to the dialysate (Fig. 6, lanes 1 and 4), indicating that the lack of activity was due specifically to enzyme inactivation. The interaction between integrase and DCQAs, therefore, did not require a divalent cation.

Assembly of integrase-DNA complex does not alter the sensitivity of integrase to DCQAs. Many inhibitors affect the ac-

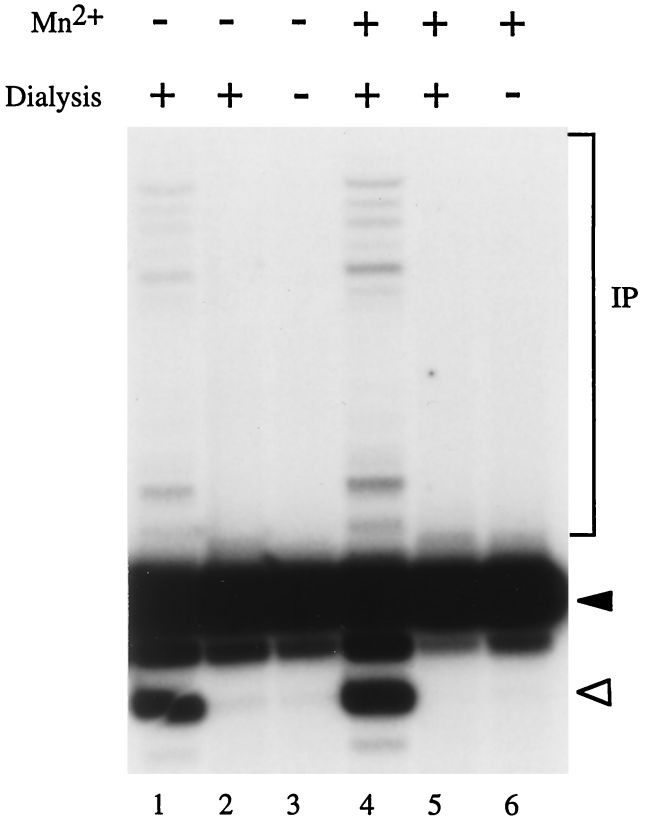

FIG. 6. Interaction between integrase and DCQAs does not require a divalent cation. HIV-1 integrase was preincubated with 1 μM (IC_{90}) L-chicoric acid in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 10 mM $MnCl_2$ at 37°C for 30 min. An aliquot of the reaction mixture was dialyzed against 2× 80 ml of buffer D at 4°C for 8 h (lanes 2 and 5), while an equal aliquot was kept at 4°C for 8 h (lanes 3 and 6). Buffer D was identical to the reaction buffer, except that $MnCl_2$ was omitted. After dialysis, a 20-μl aliquot of the dialysate was removed, and the reaction was started by adding 5 nM U5 DNA and 10 mM $MnCl_2$ and incubating at 37°C for 30 min (lanes 2 and 5). In lane 3, an aliquot of the undialyzed sample was removed, and the U5 DNA and $MnCl_2$ were added to start the reaction. In lane 6, since the sample already contained 10 mM $MnCl_2$ and was not subjected to dialysis, the reaction was started by adding 5 nM U5 DNA only. In lanes 1 and 4, the reactions were identical to those described for lanes 2 and 5, respectively, except that a fresh 1.5 pmol of HIV-1 integrase was added to the dialysate in conjunction with U5 DNA and $MnCl_2$. The symbols have the same meanings as those in the legend for Fig. 3.

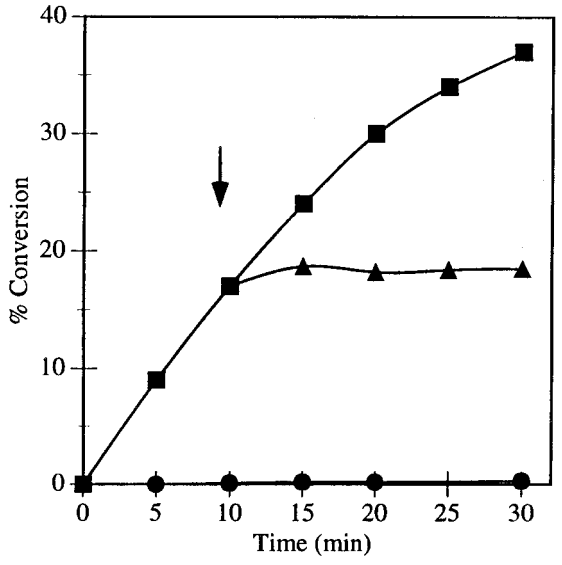

FIG. 7. Addition of L-chicoric acid halts further progression of disintegration. Disintegration reaction was carried out at 37°C by using 5 nM Y-mer substrate in the presence (■) or absence (●) of 75 nM HIV-1 integrase. The reaction was divided into two aliquots 10 min after the start of the reaction (indicated by the arrow). L-chicoric acid (3 μM [IC_{90} for disintegration]) was added to one aliquot (▲), while an equal volume of water was added to the other aliquot (■). The reaction was continued for an additional 20 min, and samples were removed at the indicated times for measuring the extent of disintegration.

tivity of integrase by disrupting the assembly of a stable complex between integrase and viral DNA and have little effect on the subsequent catalysis (27). To examine whether the action of DCQAs on integrase is affected by the assembly process, we determined the IC_{50} of the 3′-end processing reaction when an inhibitor was added during or after the assembly. With L-chicoric acid or 3,4-DCQA, the IC_{50} remained the same whether the compound was added during or after assembly (data not shown). To further confirm that the inhibition of DCQA was not altered by the assembly process, we added L-chicoric acid at its IC_{90} to a disintegration reaction 10 min after the start of the reaction (Fig. 7). In the first 10 min at 37°C, a stable integrase-DNA complex was formed (16), and the reaction proceeded normally. Addition of L-chicoric acid at 10 min immediately arrested the reaction and inhibited any further formation of the disintegration product (Fig. 7). A similar result was obtained when L-chicoric acid was added to an ongoing 3′-end processing reaction (data not shown). The results show that the assembly of integrase onto viral DNA did not alter the sensitivity of integrase to DCQAs.

DISCUSSION

Despite the essential role played by integrase in the retroviral life cycle, there is little information concerning integrase-specific inhibitors and their effects on HIV-1 replication (20, 47). Although an increasing number of integrase inhibitors have been reported in the literature, very few are specific against HIV-1 integrase and active in inhibiting replication of HIV-1 in cell culture. Also, the mechanism of action of the reported integrase inhibitors has not been well characterized.

DCQAs, a class of natural products isolated from medicinal plants from the Kallawaya of Bolivia, and their synthetic analogue, L-chicoric acid, inhibit the catalytic activities of HIV-1 integrase in vitro and replication of HIV-1 in cultured cell lines (49, 50). Kinetic analyses and dialysis experiments showed that the inhibition of HIV-1 integrase by DCQAs was irreversible and independent of divalent cations. In addition to HIV-1 integrase, DCQAs inhibit purified integrases of FIV and MLV. Analysis of the activity of inhibitors by using truncated FIV integrases suggests that the interaction occurs within the core domain of the protein. This is consistent with the previous findings that the disintegration catalyzed by only the core domain of HIV-1 integrase is inhibited by DCQAs (49) and that viruses resistant to L-chicoric acid contain a single glycine-to-serine substitution at position 140 of integrase (31). Furthermore, DCQAs have no effect on the assembly of the integrase-DNA complex, and the inhibition is likely exerted during the catalytic step. Taken together, the results suggest that DCQAs affect catalysis by interacting with the conserved core domain of retroviral integrases.

Many compounds identified as integrase inhibitors by using the biochemical assay and purified recombinant integrase, though structurally belonging to different chemical classes, contain one or more hydroxyl substituents (6, 13, 14, 22, 23, 33, 38, 43, 49, 50, 62, 63). The potency of inhibition of these chemically diverse compounds, including DCQAs, is associated with the presence of a *bis*-catechol structure, which is made up of two pairs of adjacent hydroxyls on separate benzene rings (6, 33, 38, 43, 49, 50, 62, 63). Analysis of several *bis*-catechol-containing compounds showed that they prevent assembly of integrase onto viral DNA and have no effect on the subsequent catalytic reactions (27). Since integrase has already assembled on viral DNA in the preintegration complex isolated from infected cells (60), the restricted effect of these compounds on assembly is consistent with their lack of activity on integration reactions mediated by the preintegration complex or virus replication with cultured cells (21). DCQAs, on the other hand, inhibited catalysis of integrase and had no effect on the assembly process. Therefore, the action of DCQAs is different from other *bis*-catechols tested thus far and may explain their ability to inhibit HIV-1 replication in cultured cells (49, 50).

The precise chemical mechanism by which DCQAs inactivates integrase is still not known. Based on the irreversible nature of inhibition and the structure of the active compounds, we hypothesized that it is due to oxidation-reduction. All the active compounds, including L-chicoric acid, contain a *bis*-catechol moiety (49, 50). Under aerobic conditions, the catechol can undergo 2-electron oxidation to form *ortho*-quinone (56), which is an electrophile and is highly reactive (40). DCQAs may irreversibly inhibit integrase by forming a covalent adduct. For instance, the sulfhydryl group of a cysteine residue can mediate a nucleophilic Michael addition (24) to the *ortho*-quinone moiety forming a DCQA-integrase adduct and disrupting the catalytic function of integrase. Alternatively, the *ortho*-quinone can undergo a futile redox cycling, producing oxidative stress and generating active oxygen species, such as superoxide and hydroxyl radicals (12). Consistent with the aforementioned hypothesis is the observation that replacement of the cysteine at position 65 of HIV-1 integrase by alanine increased the IC_{50}s of L-chicoric acid and 3,4-DCQA fourfold (12a). The role of the redox reaction in the mechanism of inhibition of DCQAs can be better defined by determining the change in IC_{50}s when the reaction is carried out under anaerobic conditions or in the presence of antioxidants or when the compound is preincubated under oxidizing conditions before the addition of integrase.

HIV-1 replication in vivo is continuous and highly productive (29, 45, 61). Because of the rapid turnover and high mutation rate, viral escape from monotherapy is an unavoidable consequence of the generation of viral diversity (11). By the

same token, any means of reducing viral load in an infected individual will be beneficial, as evidenced by the success of combination therapy involving reverse transcriptase and protease inhibitors (3, 48). A useful strategy for expanding the current arsenal against HIV-1 may involve development of inhibitors that are specifically directed against additional new targets, such as integrase (44, 48). Such a combination may further improve the efficacy of existing chemotherapeutic regimens while reducing their toxic side effects and retarding the emergence of drug-resistant viruses. The activity and specificity of DCQAs will be useful in understanding the biological function and biochemical property of integrase and are promising leads for the development of a new class of anti-HIV drugs.

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