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Inhibitors of HIV-1 replication that inhibit HIV integrase

(AIDS/therapeutics/natural products/chicoric acid)

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ABSTRACT **HIV-1 replication depends on the viral enzyme integrase that mediates integration of a DNA copy of the virus into the host cell genome. This enzyme represents a novel target to which antiviral agents might be directed. Three compounds, 3,5-dicaffeoylquinic acid, 1-methoxyoxalyl-3,5-dicaffeoylquinic acid, and L-chicoric acid, inhibit HIV-1 integrase in biochemical assays at concentrations ranging from 0.06–0.66 μg/ml; furthermore, these compounds inhibit HIV-1 replication in tissue culture at 1–4 μg/ml. The toxic concentrations of these compounds are fully 100-fold greater than their antiviral concentrations. These compounds represent a potentially important new class of antiviral agents that may contribute to our understanding of the molecular mechanisms of viral integration. Thus, the dicaffeoylquinic acids are promising leads to new anti-HIV therapeutics and offer a significant advance in the search for new HIV enzyme targets as they are both specific for HIV-1 integrase and active against HIV-1 in tissue culture.**

Like all retroviruses, HIV requires integration into the host chromosome for productive infection (1–3). The protein that catalyzes the integration of viral DNA into the host chromatin is integrase (IN) (4). After entry of the retrovirus into susceptible host cells, a double-stranded DNA copy of the RNA genome is synthesized by the viral enzyme, reverse transcriptase (RT). After reverse transcription, the resulting viral DNA exists as part of a large stable nucleoprotein complex that includes all the machinery required for integration (5–7). Formation of integration complexes presumably is critical for maintenance of a stable association between viral DNA and integration machinery, as well as for transport of viral DNA into the nucleus before integration (8). After nuclear entry, the viral DNA is covalently joined to a host chromosome, forming the provirus. Retroviruses depend on integration for efficient replication and for maintenance of a stably infected state. Indeed, recent reports have demonstrated that mutants of HIV-1 incapable of integration do not lead to production of progeny virions (9–12). Once integrated, the proviral DNA is transmitted as an integral element of the host genome. The IN from HIV-1 was recently crystallized (13), and rational design of IN inhibitors for use as potential anti-HIV therapeutics is underway.

Despite the critical role played by IN in the retroviral life cycle, there is little information concerning chemical compounds that show selective inhibition against the catalytic activities of IN. The major classes of IN inhibitors that have been reported to date include aurintricarboxylic acid (14) and cosalene analogues (15), caffeic acid phenylethyl ester (CAPE) (16, 17), DNA-binding agents (16, 18, 19), topoisomerase inhibitors (16), and *bis*-catechols (20). A majority of the compounds reported thus far are not selective for IN. Aurintricarboxylic acid and related compounds also inhibit RT and other phosphoryltransferases (14). Inhibition of IN by DNA-binding agents and topoisomerase inhibitors is relatively weak and nonselective. Importantly, most of the information on IN inhibitors is derived from *in vitro* experiments using purified IN and a protective effect of IN inhibitors against HIV infection in tissue culture is either undetectable (20) or has not been examined. Furthermore, the mechanisms of action for any of these compounds in inhibiting IN has not been studied.

In this report it is shown that two dicaffeoylquinic acids (DCQAs), 1-methoxyoxalyl-3,5-dicaffeoylquinic acid (1-MO-3,5-DCQA) and 3,5-dicaffeoylquinic acid (3,5-DCQA), obtained from medicinal plants, as well as one synthetic analogue, L-chicoric acid, are potent inhibitors of HIV-1 IN in biochemical assays and also inhibit HIV-1 replication *in vitro* at concentrations of approximately 1–4 μg/ml. Several smaller compounds that together comprise the basic chemical components of the DCQAs (quinic acid, caffeic acid, and 5-caffeoylquinic acid) do not inhibit HIV infection *in vivo* nor do they inhibit the integration reaction *in vitro*. Although structurally similar to IN inhibitors reported previously (16), they are unique compounds and inhibit HIV replication at nontoxic concentrations. These new IN inhibitors offer powerful tools for dissecting the basic mechanism of HIV integration and may lead to promising new therapies in the treatment of AIDS.

MATERIALS AND METHODS

DCQAs and Analogues. Both the caffeic acid (M_r = 180) and the 5-caffeoylquinic acid (chlorogenic acid) (M_r = 353) were isolated from *Achyrocline satureioides* and along with quinic acid represent the structural components of the DCQAs. All three of these compounds were also purchased (Aldrich). The 3,5-DCQA (M_r = 516) was isolated from an aqueous extract of *Baccharis genistelloides*. L-chicoric acid (M_r = 474) was synthesized from the diphenylmethyl ester of L-tartaric acid by esterification with the bis-*O*-carboxymethylcaffeoyl chloride according to the method described (21). The protecting ester groups were removed by sequential base and acid hydrolyses. The final product was purified by column chromatography on a Sephadex LH20 column. The 1-MO-3,5-DCQA (M_r = 602) was isolated from an aqueous extract of *A. satureioides*. The purities of 1-MO-3,5-DCQA, 3,5-DCQA, and L-chicoric acid were confirmed by high-pressure liquid chromatography and the structures determined by ^1H and ^{13}C nuclear magnetic resonance (NMR) analysis. The structures of 3,5-DCQA and L-chicoric acid were confirmed by comparison with literature values (21, 22). 1-MO-3,5-DCQA is a new compound and gives a matrix-assisted laser desorption ionization (MALDI) mass spectrum with M − H = 601 consistent with $C_{28}H_{26}O_{15}$ and NMR spectra in $C^2H_3O^2H$ that display, in addition to the aromatic and olefinic resonances of two caffeoyl residues, the

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Abbreviations: DCQA, dicaffeoylquinic acids; 3,5-DCQA, 3,5-dicaffeoylquinic acid; 1-MO-3,5-DCQA, 1-methoxyoxalyl-3,5-dicaffeoylquinic acid; IN, integrase; RT, reverse transcriptase; HIV_{LAI}, the LAI isolate of HIV-1; TI, therapeutic index.

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following: ^{13}C-NMR: δ = 174.1, 170.3, 168.6, 168.3, 167.7 [C = 0], 80.5 [C-1], 73.5 [C-3], 71.6 [C-4], 71.1 [C-5], 37.7 [C-6], and 32.9 [C-2]; ^{1}H-NMR: δ = 5.52 [bq, J = 3.4, 3.4, 3.4; H-3eq], 5.43 [ddd, J = 9.7, 9.7, 4.1, H-5ax], 3.96 [dd, J = 9.4, 3.5, 4-Hax], 2.73 [dd, J = 15.1, 2.5, H-2eq], 2.62 [dd, J = 12.8, 2.0, H-6eq], 2.48 [dd, J = 15.0, 2.0, H-2ax] and 2.02 [dd, J = 12.0, 10.0, H-6ax]. The above NMR assignments were consistent with the appropriate attached proton test (APT), C,H-heteronuclear correlation (HETCOR), and H,H-homonuclear correlated spectroscopy (COSY) NMR experiments.

Cells and Virus. All cell lines, including productively infected cells and the MT-2 target cell line, were grown at 37°C in RPMI 1640 medium containing Hepes and supplemented with 2 mM L-glutamine and 12% fetal bovine serum (growth medium). The LAI isolate of HIV-1 (HIV_{LAI}) was grown in H9 cells and filter-clarified before use in infectivity assays (23). HIV_{LAI} was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD).

Inhibition of HIV-1 *in Vivo*. The cell toxicity profile was measured using two-fold dilutions of each compound on MT-2 cells. Cell viability compared with solvent-treated controls was measured 48 hr after addition of the compound as described (23). The nontoxic dose was defined as that concentration where compound inhibited growth of cells by 50% or less (LD_{50}). Anti-HIV-1 activity was measured as 50% protection against HIV-1-induced cytopathic effect 72 hr after addition of virus (ED_{50}). In triplicate, HIV_{LAI} was preincubated with dilutions of the compound for 1 hr before addition of MT-2 cells as described (23). Cells and virus were then incubated for 3 days and harvested for cytopathic effect.

Confirmation of Anti-HIV Effect by RT Release and Immunofluorescence Assay. L-chicoric acid (100 μg) in 0.5 ml of growth medium was added to an equal volume of filter-clarified HIV_{LAI} (1–5 × 10^6 infectious virions) in triplicate wells of a 24-well tissue culture plate. After 1 hr at 37°C, 1 ml of MT-2 cell suspension (5 × 10^5 cells/ml) was added and cells were incubated at 37°C for 12 hr. Supernatant was removed and cells were recultured in growth medium containing L-chicoric acid at a final concentration of 50 μg/ml. Supernatants and 50% of the cells were removed at the indicated timepoints and assayed for RT release and HIV protein synthesis by immunofluorescence assay. RT in polyethylene glycol (PEG) 6000-precipitated supernatant was assayed using poly(rA)-oligo(dT) template and [^{3}H]dTTP by a modification (24) of the method of Poiesz *et al.* (25). The percentage of methanol-acetone fixed cells positive for HIV antigens was determined using a human polyclonal anti-HIV-1 pooled immunoglobulin fraction and a fluoresceinated goat anti-human IgG as described by Robinson *et al.* (24).

Inhibition of Integrase *in Vitro*. The 3′-end processing, strand transfer, and disintegration activities of IN in the presence and absence of inhibitors were assayed *in vitro* (26–28). The following oligonucleotides (Operon Technologies, Alameda, CA) were used as DNA substrates: T1 (16 mer), 5′-CAGCAACGCAAGCTTG-3′; T3 (30 mer), 5′-GTCGACCTGCAGCCCAAGCTTGCGTTGCTG-3′; V2 (21 mer), 5′-ACTGCTAGAGATTTTCCACAT-3′; V1-T2 (33 mer) 5′-ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC-3′; C220 (21 mer), 5′-ATGTGGAAAATCTCTAGCAGT-3′; B2–1 (19 mer), 5′-ATGTGGAAAATCTCTAGCA-3′. The oligonucleotides were purified by electrophoresis through a 15% denaturing polyacrylamide gel. Oligonucleotides T1, C220, and B2–1 were labeled at the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq; Amersham, Arlington Heights, IL). The substrate for 3′-end processing and strand transfer reactions, which corresponds to the terminal 21 nucleotides of the U5 end of viral DNA, was prepared by annealing the labeled C220 strand with its complementary oligonucleotide, V2. The preprocessed substrate, which resembles the viral U5 end after 3′-end processing and was used to assay only strand transfer activity, was prepared by annealing the labeled B2–1 strand with the V2 strand. The Y substrate for assaying disintegration activity, the Y-oligomer, was prepared by annealing the labeled T1 strand with oligonucleotides T3, V2, and V1/T2 (26, 28). In a 20-μl volume, the DNA substrate (0.1 pmol) was incubated with recombinant IN for 30 min at 37°C in a buffer containing a final concentration of 20 mM Hepes (pH 7.5), 10 mM DTT, 0.05% Nonidet P-40, and 10 mM $MnCl_2$. The reaction was stopped by the addition of 18 mM EDTA. Reaction products were heated at 90°C for 3 min before analysis by electrophore-

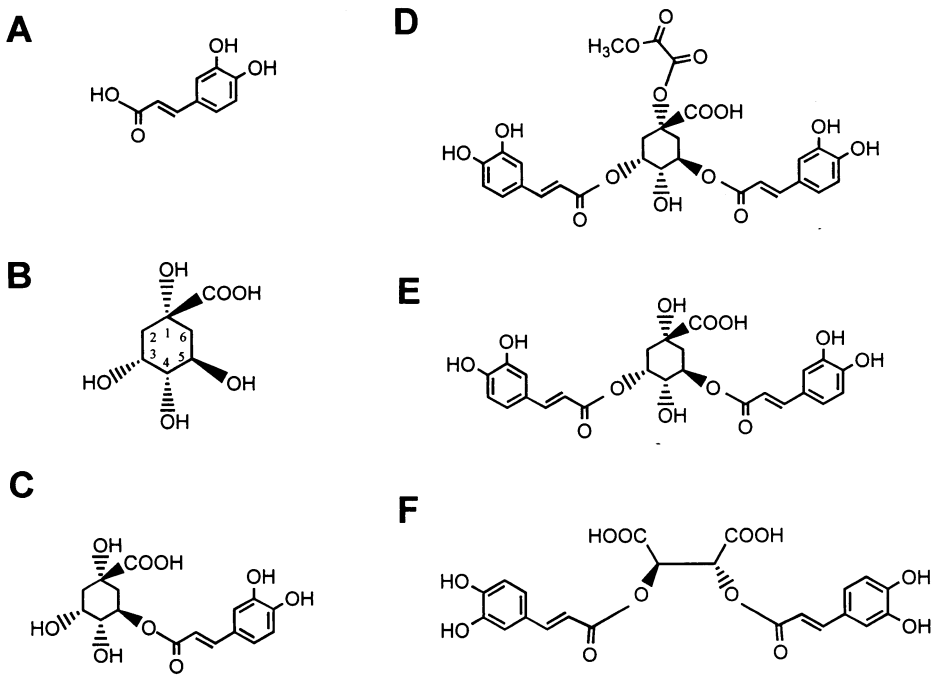

FIG. 1. Compound A is caffeic acid; compound B is the quinic acid core; compound C is the 5-caffeoylquinic acid, chlorogenic acid; compound D is 1-MO-3,5-DCQA; compound E is 3,5-DCQA; and compound F is L-chicoric acid.

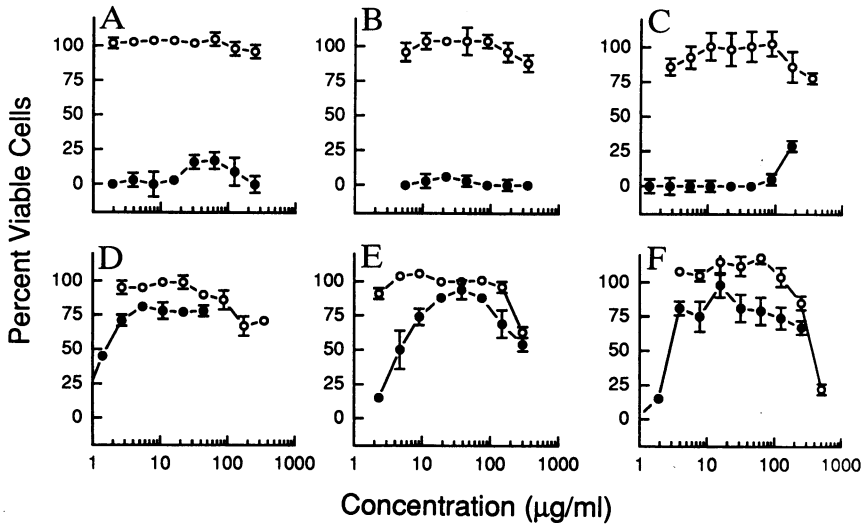

FIG. 2. Anti-HIV and cell toxicity profiles of DCQAs. Cell viability (○) and anti-HIV-1 (●) concentrations of each compound are illustrated. Error bars indicate one SD of triplicate samples. Compounds tested include: (*A*) caffeic acid; (*B*) quinic acid; (*C*) chlorogenic acid; (*D*) 1-MO-3,5-DCQA; (*E*) 3,5-DCQA; (*F*) L-chicoric acid.

sis on a 15% polyacrylamide gel with 7 M urea in Tris-borate-EDTA buffer. Inhibitor (1 μl) at various concentrations in solvent or solvent alone was added to 19 μl of each reaction mixture containing buffers, labeled-oligonucleotides, and enzyme. All compounds were tested at concentrations of 0.005, 0.05, 0.5, and 5 μg/ml for inhibition of strand transfer and 3′-end processing and at concentrations of 0.05, 0.5, 5, and 50 μg/ml for inhibition of disintegration. The IC_{50} for each reaction was obtained using nonlinear regression to fit the following logistic curve model to the dose-response data:

$$\text{Percent inhibition} = \frac{100}{1 + \left[\frac{\text{conc.}}{IC_{50}}\right]^n}$$

where conc. is equal to the concentration of the compound and n = Hill slope.

RESULTS

The Kallawaya of Bolivia have a rich ethnopharmacologic history dating over 1000 years (29). To search for new anti-HIV agents, medicinal plants from the Kallawaya have been collected, dried, and fractionated according to the protocol of the National Cancer Institute (30, 31); fractions were then tested for anti-HIV activity *in vitro*. Following this protocol, two compounds, 3,5-DCQA and 1-MO-3,5-DCQA, were isolated from *B. genistelloides* and *A. satureioides*, respectively. The structure of these two compounds are shown in Fig. 1. The cell toxicity of the 3,5-DCQA and 1-MO-3,5-DCQA were >150 μg/ml and >350 μg/ml, respectively. These concentrations inhibited growth of a T-lymphoblastoid cell line, MT-2 (23), by less than 50%, the LD_{50}, at all concentrations tested. No true LD_{50} was obtainable as the maximum toxicity was less than 50%; thus, the values given are the maximum obtainable concentration of drug in aqueous media. The anti-HIV activity of the two compounds against HIV_{LAI} *in vitro* was 1 μg/ml and 4 μg/ml, respectively, as determined by protection of 50% of MT-2 cells from HIV-induced cytopathic effect (ED_{50}). These results yielded a therapeutic index of >150 for 3,5-DCQA and >87 for 1-MO-3,5-DCQA, calculated as the inverse ratio of the ED_{50} to the LD_{50} as described (23, 32). The cell toxicity and anti-HIV profiles from a representative experiment are illustrated in Fig. 2. A synthetic analogue of these compounds, L-chicoric acid (Fig. 1), was also tested and had a similar pattern of activity. The LD_{50} for L-chicoric acid was 333 μg/ml and the ED_{50} was 2 μg/ml, giving a therapeutic index of 166. Representative cell toxicity and anti-HIV profiles for this compound from one experiment are illustrated in Fig. 2. Three likely precursor molecules of the DCQAs, chlorogenic acid, caffeic acid, and quinic acid, were also tested for anti-HIV activity and none of the three had any activity against HIV-1 *in vitro* (Fig. 2). The anti-HIV activity of the L-chicoric acid was confirmed by measuring both RT release and HIV protein synthesis (by immunofluorescence) (Table 1). In addition, in separate experiments 3,5-DCQA inhibited HIV-1 RT release by 84% at 9 μg/ml and 1-MO-3,5-DCQA inhibited HIV-1 RT release by 90% at 50 μg/ml (data not shown). Thus, all three of compounds inhibited HIV-1 replication by nearly 100% at nontoxic concentrations.

The mechanism of action of the DCQAs and L-chicoric acid was determined to be inhibition of IN. HIV-1 IN can catalyze

Table 1. Inhibition of HIV-1 infection *in vitro* by L-chicoric acid

	48 hr		72 hr	
Compound	IFA (% positive)	RT (cpm/ml)	IFA (% positive)	RT (cpm/ml)
L-chicoric acid	10	0 (203)	40	2,562 (1,856)
Caffeic acid	70	41,640 (4,390)	100[L]	636,899 (39,772)
Chlorogenic acid	70	44,640 (10,599)	100[L]	927,459 (122,169)
Water (HIV control)	70	43,731 (68)	100[L]	767,760 (39,225)

Filter-clarified HIV_{LAI} (500 μl) (1-5 × 10^6 infectious virions) was preincubated with each compound at a final concentration of 200 μg/ml for 1 hr before the addition of 5 × 10^5 MT-2 cells in a final volume of 2 ml (final concentration of compound 50 μg/ml). Virus was removed 24 hr later and cells resuspended in media containing 50 μg/ml of compound. Cells were harvested for RT and IFA at the indicated times after initial inoculation of virus (48 or 72 hr). RT values are means of triplicate infections; values in parentheses are one SD. RT increases in MT-2 cells are logarithmic at lysis (24). L, lysed.

three separate reactions. The first activity is the removal of two nucleotides from the 3′ end of the viral long terminal repeat. This activity, called 3′-end processing, leads to a terminal CA on the 3′ end of the long terminal repeat and a viral genome that is reduced by two nucleotides at both 3′ ends (33–35). The second reaction catalyzed by IN is the ATP-independent joining of the processed 3′ end of the viral long terminal repeat to the 5′ phosphate of the host DNA in a reaction referred to as strand transfer (36, 37). The final reaction demonstrated *in vitro* is the reverse of strand transfer and is called disintegration (27). These three reactions can be independently measured in biochemical assays using synthetic oligonucleotides and recombinant IN protein produced in *Escherichia coli* (26–28) and are illustrated in Fig. 3*A*.

The three active compounds, 3,5-DCQA, 1-MO-3,5-DCQA, and L-chicoric acid, inhibited HIV IN in all three reactions as illustrated in Fig. 3. The L-chicoric acid was the most active with an IC_{50} in the HIV 3′-end processing (Fig. 3*B*) and strand transfer (Fig. 3*C*) reactions of 0.06–0.07 μg/ml. The two natural products, 3,5-DCQA and 1-MO-3,5-DCQA, had IC_{50}s of ≈0.3 μg/ml in the 3′-end processing and strand transfer reactions. All three compounds had less activity in the disin-

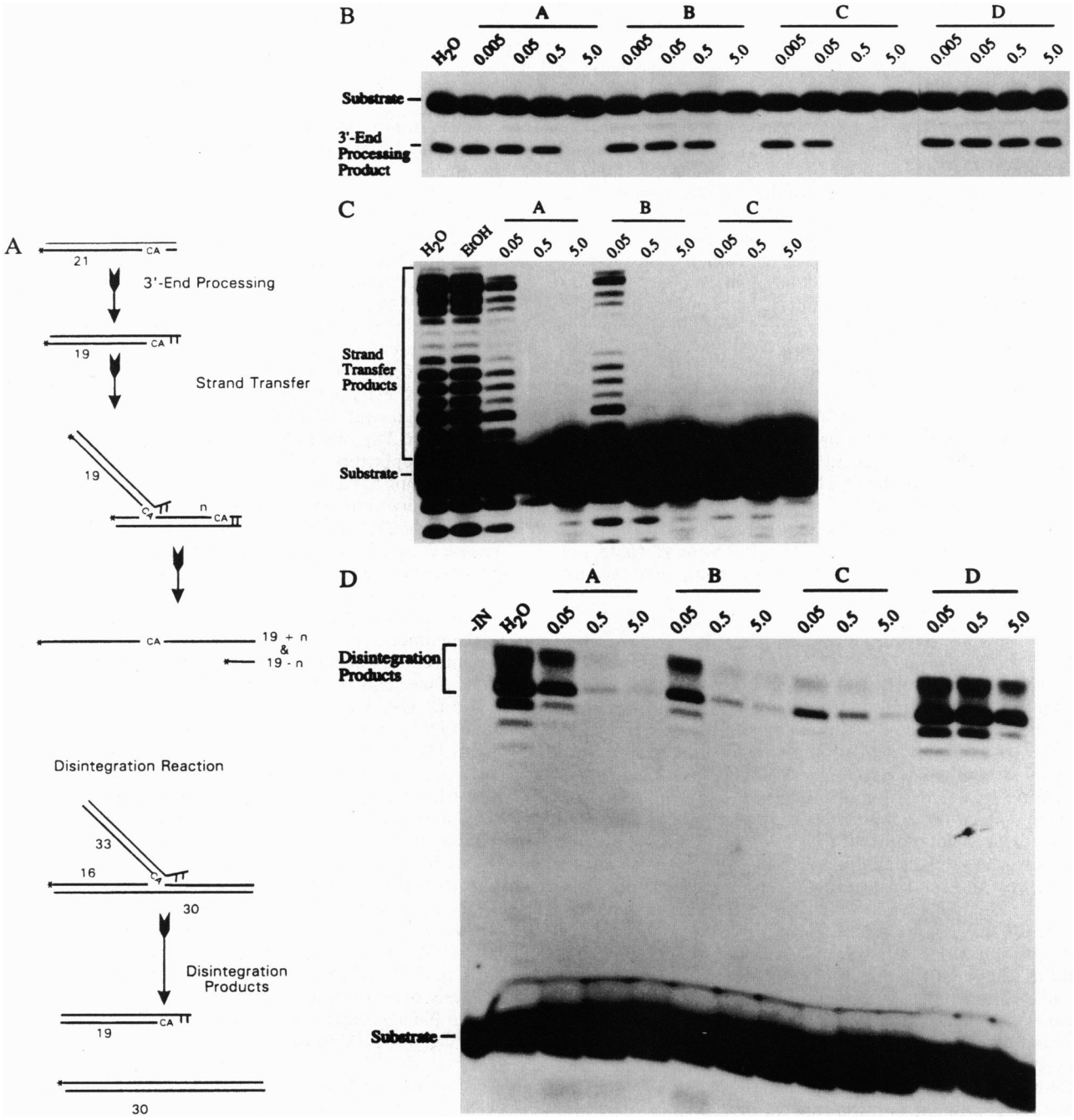

FIG. 3. IN reactions are inhibited by DCQAs and a synthetic analogue but not likely precursor molecules. (*A*) The three separate reactions catalyzed by HIV IN and their corresponding reaction products. Illustrated reactions: (*B*) 3′-end processing; (*C*) strand transfer; (*D*) disintegration. For all three panels, compound A was 3,5-DCQA; compound B was 1-MO-3,5-DCQA; compound C was the synthetic analogue, L-chicoric acid; compound D was a nonactive, likely precursor of the DCQAs. (*B*) Chlorogenic acid. (*C* and *D*) Caffeic acid. All compounds were tested at concentrations of 0.005, 0.05, 0.5, and 5 μg/ml for inhibition of strand transfer and 3′-end processing and at concentrations of 0.05, 0.5, 5, and 50 μg/ml for inhibition of disintegration. The activity of IN is illustrated by solvent lanes labeled H_2O and EtOH for water and 10% ethanol solvents, respectively.

Table 2. Summary of the anti-HIV and anti-HIV IN activities of the DCQAs, their likely precursors, and L-chicoric acid

Compound	LD_{50} (μg/ml)	ED_{50} (μg/ml)	Inhibition of HIV-1 IN (IC_{50}, μg/ml)		
			3′-end processing	Strand transfer	Disintegration
Quinic acid	>625	>350	ND	ND	>50
Chlorogenic acid	>350	>176	>50	>50	>50
3,5-DCQA	>150	1	0.33	0.34	0.66
1-MO-3,5-DCQA	>350	4	0.27	0.26	0.57
L-chicoric acid	333	2	0.07	0.06	0.15

Each compound was tested for inhibition of MT-2 cell growth as described by Montefiori *et al.* (23). Values listed as > represent the maximum attainable concentration of compound in growth medium although less than 50% inhibition of growth occured. Values for both ED_{50} and LD_{50} are given as μg/ml and represent triplicate samples from no less than three separate experiments. The IC_{50} for the three separate IN reactions were determined in a blinded fashion using quadruple reactions and 10-fold dilutions of compound over a four-dilution curve and are given in μg of drug per ml. Percent inhibition was calculated on a phosphoimager. For strand transfer reactions, the percent inhibition was determined by scanning all of the potential strand transfer products rather than selected bands. The three separate IN reactions are described.

tegration reaction (Fig. 3*D*). Previous work has shown that the disintegration reaction can be mediated by the core domain unlike either 3′-end processing or strand transfer reactions. In the disintegration reaction, IC_{50}s for the compounds were $\approx$0.6 μg/ml for both 3,5-DCQA and 1-MO-3,5-DCQA and 0.15 μg/ml for L-chicoric acid. Negative controls for these reactions, chlorogenic acid and quinic acid, had little activity against HIV-1 IN in these reactions and at concentrations much greater than the active agents. The reactions described here were carried out in the presence of Mn^{2+} as the divalent cation. The DCQAs and L-chicoric acid also inhibited IN-mediated reactions in the presence of Mg^{2+} (data not shown). The DCQAs were specific for HIV-1 IN in that they only weakly and nonspecifically inhibited HIV surface glycoprotein (M_r 120,000) binding to CD4 or HIV-1 RT *in vitro* at concentrations that were inconsistent with their observed ED_{50} in tissue culture (data not shown). Likewise, the compounds were tested for activity against RNase H both in a commercially available scintillation assay and in a gel-shift assay; the compounds had no activity against HIV-1 RNase H (data not shown). The inhibitory activity of these five compounds against HIV-1 IN are summarized in Table 2.

DISCUSSION

After attachment, internalization, and uncoating of a retrovirus particle, reverse transcription leads to a double-stranded DNA molecule. This double-stranded molecule must integrate into the host cell DNA for proper transcription, translation, and viral assembly. The protein that mediates integration is IN. Therefore, HIV-1 IN is an essential protein in the retroviral lifecycle. Of the three reactions catalyzed by IN, the disintegration assay is an important indication of where these compounds work as the disintegration reaction has been shown to require only the central core domain of the IN protein (38), unlike the 3′-end processing and strand-transfer reactions that require the full-length IN (38). Since these compounds inhibit disintegration, it is likely they act at the core domain which contains the highly conserved catalytic motif D, D,-35-E (aspartate, aspartate, 35 amino acid spacer, glutamate) (9). Although not illustrated in these results, the active compounds also inhibited feline immunodeficiency virus IN (39), a genetically divergent IN protein. Finally, these compounds also inhibited disintegration mediated by amino acids 50–234 of the HIV-1 IN, containing only the core catalytic domain (data not shown). Taken in concert, these results strongly implicate interaction of the DCQAs and L-chicoric acid with the catalytic domain of HIV-1 IN for anti-HIV activity. However, we cannot rule out an interaction between DNA and these compounds; however, their selectivity in tissue culture suggests that these compounds would require a predilection for HIV DNA over cellular DNA.

These results suggest that ethnopharmacologic agents, derived from traditional sources, may be rich sources of compounds with unique antiviral properties. The compounds reported herein are the first nonoligonucleotide compounds reported that inhibit HIV-1 IN and HIV replication *in vitro* at concentrations consistent with inhibition of IN as a mechanism of action. Their activities against HIV-1 IN in cell-free assays is as great as the activity of any compounds previously reported to inhibit HIV-1 IN (14–20). Two other IN inhibitors have been reported that block HIV replication in tissue culture. One of these, curcumin (40), blocks HIV replication at concentrations significantly higher than the concentration capable of blocking HIV IN activity. Thus, curcumin would have to be concentrated over 10-fold within the cell to act through this mechanism. The other compound reported to block HIV IN and HIV replication, suramin (41), acts through inhibition of gp120 binding to CD4; suramin has a net negative charge of −6 and likely does not get into cells to act against IN at all. Unlike suramin, the DCQAs are not highly charged and, indeed, their observed anti-HIV activity is unrelated to charge as the chlorogenic acid has the same net charge as the DCQAs (Fig. 1) but is not effective against HIV IN or against HIV in tissue culture. Therefore, the DCQAs are more potent inhibitors of IN than others reported and, in addition, block viral replication in tissue culture at concentrations consistent with inhibition of IN as a mechanism of action.

The DCQAs may prove useful in further defining the active site of IN by cocrystallization of these inhibitors with recombinant HIV-1 IN and other retroviral IN proteins. Although these compounds have TI of less than 200, these plants have been used therapeutically by the Kallawaya for centuries (29) without significant toxicities. Such usage suggests that the DCQAs might be used as a successful solo or adjunct therapy in AIDS. Whether the pure compounds could be used in patients without the synthesis of more active, less toxic analogues remains to be evaluated. Regardless of the ultimate clinical utility of the DCQAs in the treatment of HIV infection and AIDS, they may prove important in understanding the function of HIV-1 IN at the molecular level, thus leading to the synthesis of more selective and potent IN inhibitors in the future. Finally, these compounds are useful because they are active against HIV in tissue culture.

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