Curcumin Analogs with Altered Potencies against HIV-1 Integrase as Probes for Biochemical Mechanisms of Drug Action

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We have previously reported the inhibitory activity of curcumin against human immunodeficiency virus type one (HIV-1) integrase. In the present study, we have synthesized and tested analogs of curcumin to explore the structure−activity relationships and mechanism of action of this family of compounds in more detail. We found that two curcumin analogs, dicaffeoylmethane (**6**) and rosmarinic acid (**9**), inhibited both activities of integrase with IC_{50} values below 10 μM. We have previously demonstrated that lysine 136 may play a role in viral DNA binding. We demonstrated equivalent potencies of two curcumin analogs against both this integrase mutant and wild-type integrase, suggesting that the curcumin-binding site and the substrate-binding site may not overlap. Combining one curcumin analog with the recently described integrase inhibitor NSC 158393 resulted in integrase inhibition which was synergistic, reflective of drug-binding sites which may not overlap. We have also determined that these analogs can inhibit binding of the enzyme to the viral DNA but that this inhibition is independent of divalent metal ion. Furthermore, kinetic studies of these analogs suggest that they bind to the enzyme at a slow rate. These studies can provide mechanistic and structural information which may guide the future design of integrase inhibitors.

Introduction

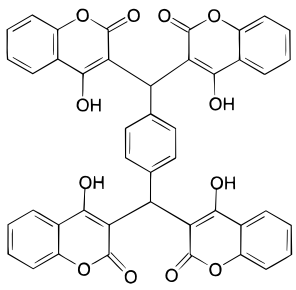
Several key enzymes in the replication cycle of the human immunodeficiency virus (HIV) can be targeted for chemotherapeutic intervention, most notably, reverse transcriptase and protease.[1] Research is now in progress to develop clinically active agents against other proteins in the viral life cycle. One such target is the viral integrase protein.[2]

Efficient replication of retroviral DNA requires establishment of the proviral state—i.e., the integration of a DNA copy of the viral genome, synthesized by reverse transcriptase, into a chromosome of the host cell. Integration is catalyzed by the viral integrase protein. Prior to integration, two nucleotides are excised from each 3′-end of the linear, blunt-ended, viral DNA. This 3′-processing reaction exposes the 3′-hydroxyl of a CA dinucleotide which is conserved among all retroviruses. Each of these 3′-hydroxyl ends of the viral DNA is then joined to chromosomal DNA in the subsequent DNA strand transfer step. DNA strand transfer is an isoenergetic transesterification reaction. HIV-1 integrase catalyzes a nucleophilic attack of each 3′-hydroxyl group at the processed viral ends on a pair of phosphodiester bonds staggered by five base pairs in the target DNA. Completion of the integration process requires removal of the two unpaired nucleotides at the 5′-ends of the viral DNA and gap repair reactions that are thought to be accomplished by cellular enzymes.[2−4]

We and others have investigated the pharmacological activity of various compounds as inhibitors of HIV integrase.[2] A majority of the reported integrase inhibitors are polyhydroxylated aromatic compounds.[2] We have also previously reported the anti-integrase activity of curcumin.[5]

Curcuminoids, phenolic diarylheptanoids, are characteristic, yellow-coloring constituents of turmeric, the roots/rhizomes of *Curcuma aromatica* SALISB., *C. longa* L., *C. xanthorrhiza* ROXB., and *C. zedoaria* (CHRISTM.) ROSC. cultivated in tropical areas and used in traditional medicine and as spices in South and South East Asia. Besides curcumin (diferuloylmethane) (**5**), there are some closely related natural congeners, such as monodemethoxycurcumin (*p*-coumaroylferuloylmethane) (**4**) and bisdemethoxycurcumin (di-*p*-coumaroylmethane) (**2**).

The present study explores the mechanism of action of curcumin (**5**) in more detail using natural and synthetic analogs. We demonstrate equivalent potencies of curcumin analogs against wild-type integrase and an integrase protein containing a mutation in the DNA-binding domain.[6] The synergistic effects obtained using a curcumin analog in combination with another integrase inhibitor, NSC 158393,[7] are reflective of drug-binding sites which may not overlap. Binding of these analogs to the enzyme and of these enzyme−inhibitor complexes to viral DNA was examined. These studies can provide mechanistic and structural information which may guide the future design of integrase inhibitors.



NSC 158393

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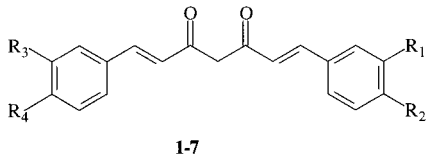

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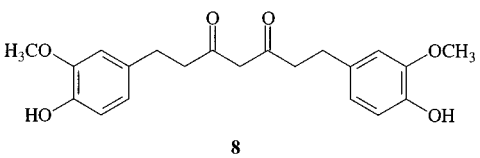

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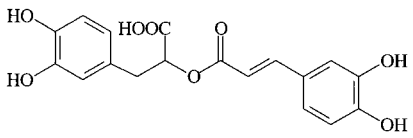

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Compound	Substituents R1	R2	R3	R4	IC50 (μM) 3-Processing	Strand Transfer
1	H	H	H	H	> 300	> 300
2[a]	H	OH	H	OH	120	80 ± 20
3	H	OCH_3	H	OCH_3	> 300	> 300
4[a]	H	OH	OCH_3	OH	140	120
5[a,b]	OCH_3	OH	OCH_3	OH	150	140
6	OH	OH	OH	OH	6.0 ± 1.5	3.1 ± 0.1
7	OCH_3	OH	OH	OH	18.0 ± 9.0	9.0 ± 3.0
8					> 300	> 300
9[c]					9 ± 7	4.0 ± 1.5

[a] Natural products, [b] Curcumin, [c] Rosmarinic Acid.

Figure 1. (A) Structures of curcumin and the eight analogs tested in this study. (B) IC_{50} values for the inhibition of the 3′-processing and strand transfer activities of HIV-1 integrase by curcumin and its analogs. IC_{50} values below 100 μM are expressed as the mean ± standard deviations obtained from three separate experiments.

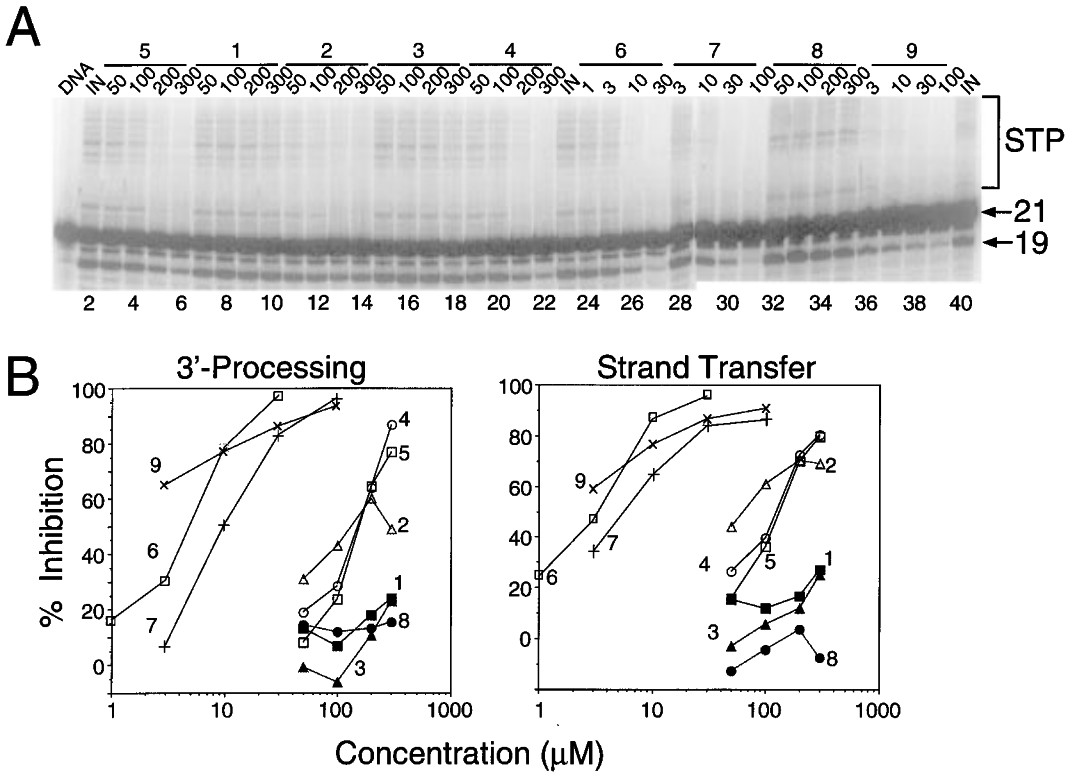

Figure 2. Effect of curcumin and its analogs on the inhibition of 3′-processing and strand transfer catalyzed by HIV-1 integrase. (A) PhosphorImager picture of a typical experiment. Drug concentrations in (μM) are indicated above each lane. The DNA strand transfer products (STP) are indicated by the bracket. The 3′-processing product (19-mer) and the DNA substrate (21-mer) are also shown. Lane 1, DNA alone; lanes 2, 23, and 40, plus integrase (IN); lanes 3−6, plus integrase in the presence of curcumin (**5**); lanes 7−10, plus integrase in the presence of **1**; lanes 11−14, plus integrase in the presence of **2**; lanes 15−18, plus integrase in the presence of **3**; lanes 19−22, plus integrase in the presence of **4**; lanes 24−27, plus integrase in the presence of **6**; lanes 28−31, plus integrase in the presence of **7**; lanes 32−35, plus integrase in the presence of **8**; lanes 36−39, plus integrase in the presence of **9**. (B) Quantitation of the dose−response shown in panel A.

Results and Discussion

Synthesis of Naturally Occurring Curcuminoids and Analogs. Curcumin (**5**) and related compounds (**1**−**4**) were prepared from different substituted benzaldehydes and 2,4-pentanedione−boric anhydride in EtOAc in the presence of tributyl borate and *n*-butylamine using the original synthetic strategy of Pabon.[8] Creating a boron complex of 2,4-pentanedione protects the

highly acidic methylene protons from Knoevenagel condensation, and the condensation can occur at the terminal methyl groups of the diketone.[9] Methoxy groups of curcumin (**5**), which are in the ortho position of phenolic hydroxyls, were demethylated by $AlCl_3$/pyridine in CH_2Cl_2 to furnish the catechol structure.[10] By application of this method, **6** and **7** were obtained from **5**. Tetrahydroxycurcumin (**8**) was obtained by hydrogenation of **5** over Pd/C in EtOAc.[11]

Curcumin Analogs with Altered Potencies. The structures and anti-HIV-1 integrase of curcumin and the analogs used in this study are shown in Figure 1. The position and number of hydroxyl groups were altered to determine the effect(s) of these substituents. These compounds were initially tested against HIV-1 integrase in a dual 3′-processing and strand transfer assay. The results of a typical experiment are shown in Figure 2A, quantitation of the data are shown in Figure 2B, and IC_{50} values for both the 3′-processing and strand transfer activities are reported in Figure 1B.

Curcumin (**5**) (obtained from Sigma Chemical Co. and other sources) was previously found to inhibit the strand transfer activity of HIV-1 integrase with an IC_{50} of about 40 μM.[5] In the present study we purified curcumin and found this preparation to inhibit integrase with an IC_{50} about 3-fold higher. Compounds **1**, **3**, and **8** showed no activity against integrase even at concentrations of 300 μM, suggesting the importance of both the hydroxyls and the unsaturated linker present in curcumin. Compounds **2** and **4** showed potencies comparable to that of curcumin, suggesting that the methoxy groups do not play a key role in potency. Three analogs of curcumin, dicaffeoylmethane (**6**), caffeoylferuloylmethane (**7**), and rosmarinic acid (**9**), were found to be very potent. These three analogs had at least one catechol substructure.

Two of these more potent analogs, **6** and **9**, were examined for their effects on the strand transfer reaction using a "precleaved" substrate corresponding to the product of the 3′-processing reaction. Both of these analogs were able to inhibit the strand transfer activity of HIV-1 integrase in this assay (Figure 3) with IC_{50} values in the same range as those obtained using the blunt-ended oligonucleotide (Figure 2). These data are consistent with earlier findings reported for curcumin.[5]

Although curcumin has been reported to exhibit antiviral activity,[5] none of the derivatives tested in this study exhibited antiviral activity in the NCI Antiviral Drug Screen. These compounds exhibited considerable cytotoxicity (IC_{50} values ranging from 6 to 35 μM) against uninfected CEM cells.

Curcumin Analogs Bind in the Enzyme Catalytic Core. The binding site of these analogs was first examined by testing the three active analogs with an integrase deletion mutant containing only amino acids 50−212. Because deletion mutants of integrase are inactive in both 3′-processing and strand transfer [12] but can catalyze the disintegration reaction,[13] the branched Y oligonucleotide was used as the substrate (Figure 4A). Inhibition of an integrase deletion mutant which is missing both the N-terminal zinc finger and C-terminal DNA-binding domains was exhibited by **6**, **7**, and **9** (Figure 4B,C). These data suggest that these analogs bind in the enzyme catalytic core.

We have previously demonstrated that lysine 136 may play a role in viral DNA binding, [6] suggesting that this

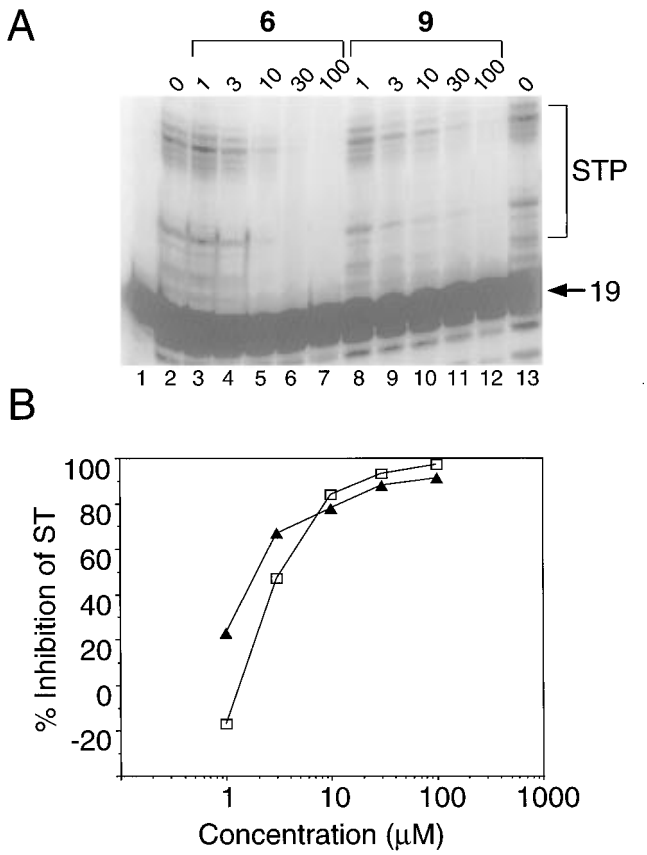

Figure 3. Inhibition of the strand transfer activity of integrase using **6** and **9**. (A) PhosphorImager picture of a typical experiment. Drug concentrations (μM) are indicated above each lane. The DNA strand transfer products (STP) are indicated by the bracket. The DNA substrate (19-mer) is also shown. Lane 1, DNA alone; lanes 2 and 13, plus integrase; lanes 3−7, plus integrase in the presence of **6**; lanes 8−12, plus integrase in the presence of **9**. (B) Quantitation of the dose−response shown in panel A.

amino acid may be located at the viral DNA-binding site. We examined whether the curcumin-binding site and the substrate-binding site may overlap by testing two analogs against both wild-type and K136E mutant integrase proteins. We found that both **6** and **9** demonstrated equivalent potencies against both proteins (data not shown). These data suggest that there is no key interaction between the positively charged side chain of Lys 136 and either of the curcumin analogs tested and that the curcumin-binding site and the substrate-binding site may not overlap. The viral substrate DNA presumably binds in the catalytic domain in close proximity to the catalytic acidic acid residues aspartate 64, aspartate 116, and glutamate 152.[4,14−16] Because the binding site of these analogs may reside in the catalytic core as suggested by the disintegration assay (Figure 4) but may not overlap with the DNA-binding site, we wanted to determine whether curcumin analogs could inhibit binding of the integrase to its substrate DNA. We also investigated whether this inhibition of binding was divalent metal ion dependent. Compound **6** was able to inhibit binding of the enzyme to its substrate DNA (Figure 5), although at 3−5-fold higher concentrations than those required for inhibition of the catalytic activity (Figures 1 and 2). Inhibition of DNA binding was observed in the presence of either manganese, magnesium, or EDTA but was more potent

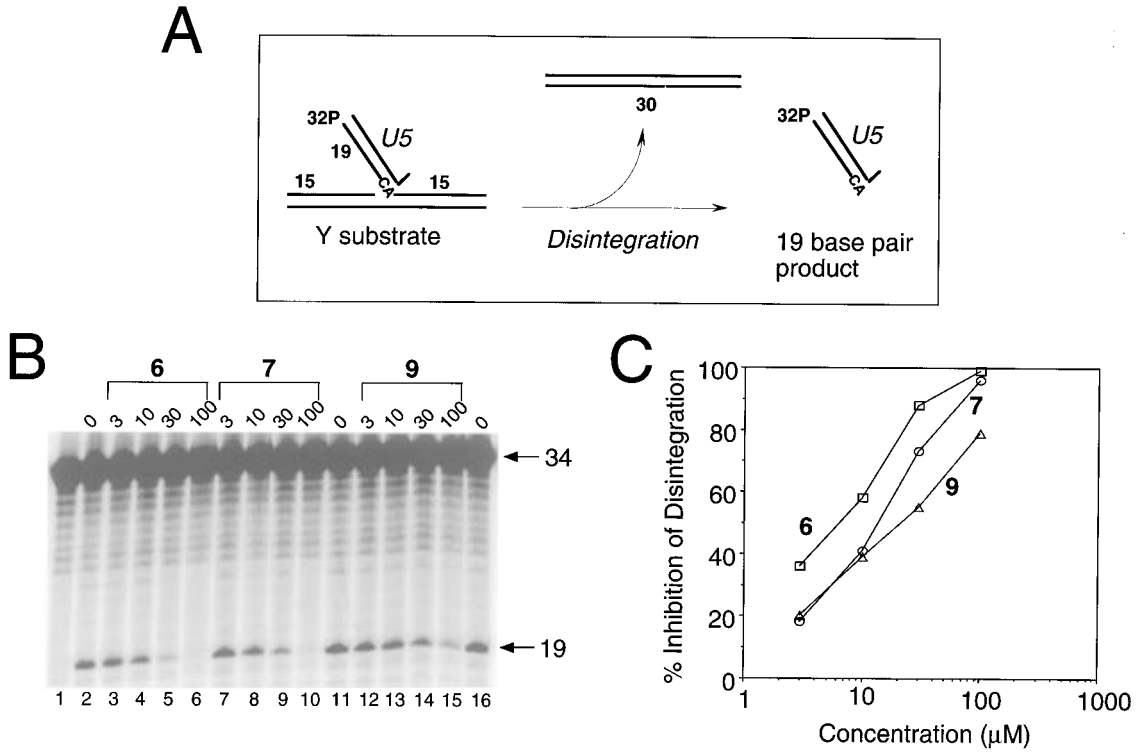

Figure 4. Inhibition of disintegration catalyzed by the integrase catalytic domain in the presence of curcumin analogs. (A) Schematic diagram of the disintegration reaction. The reverse or "disintegration" activity can be studied using a "Y oligonucleotide" in which the U5-end has been integrated into a target oligonucleotide. The integrase can catalyze a transesterification reaction, excising this U5 oligonucleotide, to generate a radiolabeled 19-mer. (B) PhosphorImager picture of a typical experiment. Compound concentrations (μM) are indicated above each lane. The DNA substrate and product are depicted by the 34-mer and 19-mer, respectively. Lane 1, DNA alone; lanes 2, 11, and 16, plus integrase; lanes 3−6, plus integrase in the presence of **6**; lanes 7−10, plus integrase in the presence of **7**; lanes 12−15, plus integrase in the presence of **9**. (C) Quantitation of the dose−response shown in panel B.

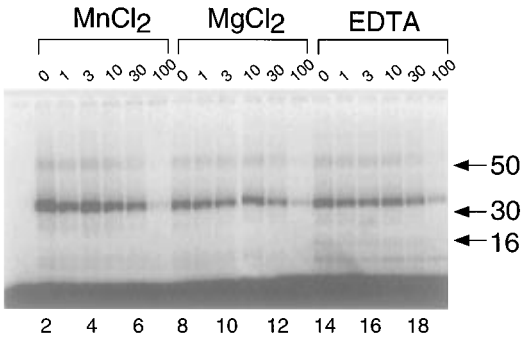

Figure 5. Inhibition of the DNA-binding activity of integrase in the presence of **6**. Molecular weight markers are shown to the right of the gel. Lanes 2−7, 8−13, and 14−19 used reaction buffer containing 2 mM $MnCl_2$, $MgCl_2$, or EDTA, respectively. Lane 1, DNA alone; lanes 2, 8, and 14, plus integrase; lanes 3−7, 9−13, and 15−19, plus integrase in the presence of the indicated concentration of **6**.

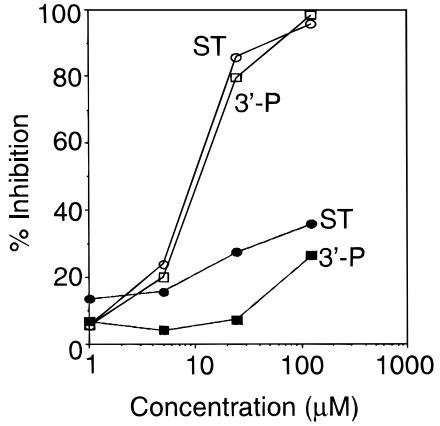

Figure 6. Differential inhibition of 3′-processing and strand transfer by compound **6** in the presence of either $MnCl_2$ or $MgCl_2$. Reactions performed in the presence of 7.5 mM $MnCl_2$ or $MgCl_2$ are depicted by the open and filled symbols, respectively. 3′-Processing (3′-P) and strand transfer (ST) are depicted by the squares and circles, respectively.

when manganese was present. Compound **6** was also able to inhibit the formation of a DNA−enzyme complex when the inhibitor was added after the addition of DNA, i.e., after stable complex formation[17] could occur (data not shown).

The inhibitory activities of these curcumin analogs against the catalytic activities of integrase were demonstrated in *in vitro* assays employing manganese (Figures 2−4). However, because the inhibition of DNA binding is not manganese dependent (Figure 5) and because integrase is likely to use magnesium *in vivo* as it does in preintegration complexes,[18−20] we investigated whether the inhibitory activity of these curcumin analogs was manganese dependent. As seen in Figure 6, the activity of **6** was abolished in magnesium compared to manganese. The apparent discrepancy between inhibition of DNA binding (Figure 5) but not catalysis (Figure 6) in buffer containing magnesium can be reconciled by the fact that the DNA binding assay measures both nonspecific and specific binding while the catalytic activity requires specific binding of the enzyme at the processing site. Therefore, in buffer containing magnesium, nonspecific binding may be affected more significantly than specific binding.

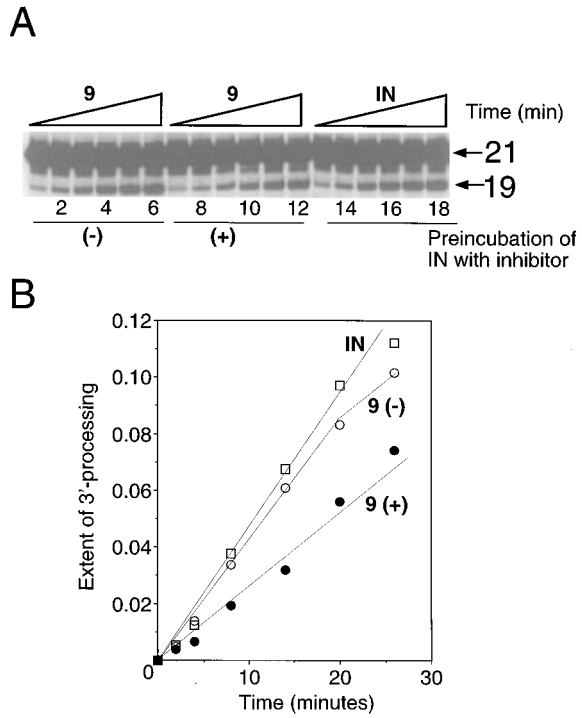

Figure 7. Kinetics of inhibition of HIV-1 integrase by **9**. PhosphorImager picture of a typical experiment. Reaction times (min) are indicated in panel B. The 3′-processing product (19-mer) and the DNA substrate (21-mer) are shown. Lanes 1−6, integrase plus **9** added at the same time as the DNA; lanes 7−12, integrase plus **9** added prior to the addition of DNA; lanes 13−18, integrase plus DNA. (B) Quantitation of the dose−response shown in panel A.

The lack of inhibitory activity of **6** in magnesium contrasts with recent findings with other integrase inhibitors such as NSC 158393 (A.M. and Y.P., unpublished data). These observations also suggest that there may be two or more distinct drug-binding sites on the enzyme. The affinity for one of these sites may be metal dependent and the other may be metal independent.

Kinetic and Mechanistic Studies of the Curcumin-Binding Site. We studied the rate of binding of **9** to wild-type integrase by examining the extent of 3′-processing catalyzed by integrase when the enzyme was or was not preincubated with inhibitor at a concentration (5 μM) close to its IC_{50}. As seen in Figure 7, the integrase control exhibited a linear increase in the extent of 3′-processing over a time range of 0−26 min. When the enzyme was preincubated for 30 min with **9**, the extent of 3′-processing was approximately 50% that of the uninhibited control. However, if the inhibitor was not preincubated with integrase prior to the start of the reaction, the slope of the line depicting the extent of 3′-processing resembled that of the uninhibited control. These data suggest that there is a slow binding of **9** to the enzyme. Alternatively, the binding of **9** may require a conformational change in the enzyme (e.g., an open or relaxed conformation).

The slow binding observed with **9** (Figure 7) was previously seen with compounds of the tyrphostin family[21] of integrase inhibitors (A.M. and Y.P., unpublished observations) and with flavones and caffeic acid phenethyl ester,[22] suggesting that the bis-catechol substructure may play a key role in the association rate of inhibitors to the integrase.

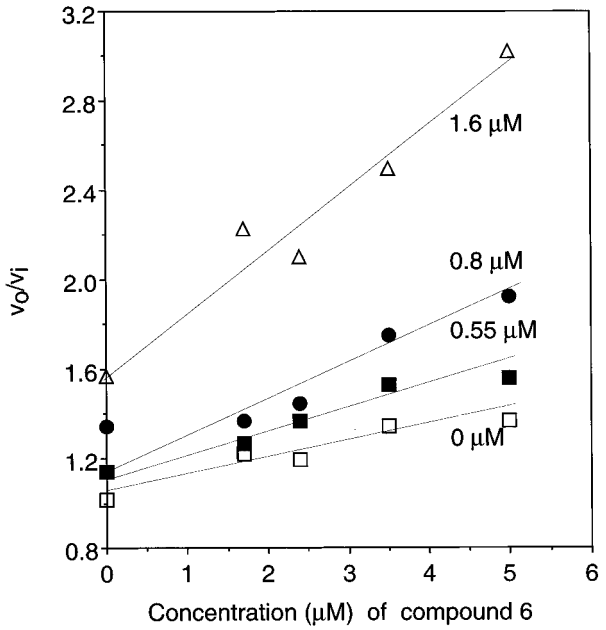

Figure 8. Yonetani−Theorell plot depicting the inhibition of the 3′-processing activity of HIV-1 integrase in the presence of both NSC 158393 and **6**. The concentration of **6** is plotted against the ratio of the rate of the uninhibited reaction divided by the rate of the inhibited reaction (v_o/v_i) for each of four combinations with NSC 158393. The concentrations of NSC 158393 are indicated to the right of each curve.

The potential for the binding site of these curcumin analogs to be distinct from or overlap with that of other integrase inhibitors was examined. The possibility of different binding sites on the enzyme would manifest itself in synergistic inhibition when a combination of the inhibitors is used, while identical or overlapping binding sites would result in mutually exclusive binding of either inhibitor. We investigated whether **6** demonstrated synergistic or mutually exclusive inhibition of integrase when used in combination with another recently described integrase inhibitor, NSC 158393.[7] The NSC 158393 consists of four 4-hydroxycoumarin rings linked by a phenyl linker and does not contain any catechol substructure. The different structure present in this inhibitor compared with **6** (Figure 1) suggested that it may not bind to the same site on the enzyme. As seen in Figure 8, the Yonetani−Theorell plot of the inhibition of 3′-processing shows nonparallel lines when the concentration of **6** is varied from 0 to 5 μM and the concentration of NSC 158393 is increased from 0 to 1.6 μM. These data suggest that these two compounds exhibit synergy in their inhibitory activity and that the both NSC 158393 and **6** can bind to integrase simultaneously and at different sites. The binding site of the curcumin family of compounds may not overlap with either the viral DNA-binding site (Figure 5) or other inhibitor-binding sites (Figure 8).

Experimental Section

Chemical Methods: **General Experimental Procedures.** ^{1}H NMR spectra were recorded on a Bruker AC 400 spectrometer. Chemical shifts are given in ppm downfield from TMS. EIMS and HRMS were obtained using MAT-711 and CH5DF Finnigan spectrometers operating at 70 eV. Elemental analyses for carbon and hydrogen were determined with a Perkin-Elmer 240C instrument, and values are within 0.4% of the theoretical values. Purity of compounds was proved by HPLC using a reverse phase Eurospher 100-C18

column; eluent MeOH:phosphoric acid, 0.5% (v/v), at 1.5 mL/min (gradient system from 65:35 to 100:0 within 35 min). Melting points were taken on a Buchi 530 melting point apparatus and are uncorrected. All experiments were monitored by thin layer chromatography using aluminum sheets coated with a 0.2 mm layer of silica gel 60 F254 (Merck art. no. 5554).

General Procedure for the Preparation of Compounds 1−5. 1,7-Bis(4-methoxyphenyl)-1,6-heptadiene-3,5-dione (3). 2,4-Pentanedione (1.0 g, 0.01 mol) and boric anhydride (0.49 g, 0.007 mol) were dissolved in EtOAc (10 mL) and stirred for 0.5 h at 40 °C. 4-Methoxybenzaldehyde (2.72 g, 0.02 mol) and tributyl borate (4.6 g, 0.02 mol) were added, and the reaction mixture was stirred for 0.5 h. After the dropwise addition of a solution of *n*-butylamine (1.1 g, 0.015 mol) in EtOAc (10 mL) over a period of 15 min, the mixture was stirred for a further 24 h at 40 °C. The mixture was hydrolyzed by the addition of 10% HCl (10 mL) and heating to 60 °C for 1 h. The organic layer was separated, and the aqueous layer was extracted three times with EtOAc. The combined organic layers were washed with water and dried over Na_2SO_4. Evaporation of the solvent left a yellow powder which was purified by column chromatography (silica gel, cyclohexane−EtOAc, 50:50 (v/v)) to give **3** as crystals from ether/hexane (2.2 g, 65%): mp 138 °C; ^{1}H NMR (DMSO-d_6) δ 3.81 (6 H, s), 6.10 (1 H, s), 6.79 (2 H, d, J = 15.8 Hz), 7.01 (4 H, d, J = 8.5 Hz), 7.60 (2 H, d, J = 15.8 Hz), 7.69 (4 H, d, J = 8.5 Hz); EIMS m/z 336 ($M^{\bullet+}$, 51). Anal. ($C_{21}H_{20}O_4$) C, H.

1,7-Diphenyl-1,6-heptadiene-3,5-dione (1): crystals from ether/hexane; mp 141−142 °C (lit.[9] mp 139−140 °C).

1,7-Bis(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione (2): crystals from ether/hexane; mp 224 °C (lit.[9] mp 223−224 °C).

1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin, 5): crystals from MeOH; mp 180−182 °C (lit.[9] mp 182−183 °C).

1-(4-Hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (4). 4 was prepared by modification of the above-described method using 4-hydroxybenzaldehyde and vanillin as aldehydes.[9] Separation of the products by thin layer chromatography (silica gel 60 PF_{254} with $CaSO_4$, cyclohexane−EtOAc, 80:20 (v/v)) using the technique of radial development (Chromatotron) gave 18% of pure **4**: mp 170−171 °C (lit.[9] mp 172−173 °C).

Demethylation of Curcumin (5). 1,7-Bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (6) and 1-(3,4-Dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (7). In a solution of **5** (5.5 g, 0.015 mol) in CH_2Cl_2 (250 mL) was suspended $AlCl_3$ (6.7 g, 0.05 mol), and pyridine (16 mL, 0.2 mol) was added dropwise under stirring. The mixture was refluxed for 24 h, cooled in ice, and acidified with diluted HCl. The aqueous layer was extracted exhaustively with EtOAc. The combined organic layers were separated and evaporated to dryness. The residue was dissolved in MeOH (5 mL) and separated by thin layer chromatography (silica gel 60 PF_{254} with $CaSO_4$, cyclohexane−EtOAc−EtOH, 55:40:5 (v/v/v)) using the technique of radial development (Chromatotron) to give 3.1 g (60%) of **6** and 0.8 g (15%) of **7**.

1,7-Bis(3,4-dihydroxyphenyl)-1,6-heptadiene-3,5-dione (6): yellow powder from $CHCl_3$; mp 306−308 °C dec; ^{1}H NMR (DMSO-d_6) δ 6.08 (1 H, s), 6.57 (2 H, d, J = 15.8 Hz), 6.79 (2 H, d, J = 8.2 Hz), 7.02 (2 H, dd, J = 8.2, 1.5 Hz), 7.08 (2 H, d, J = 1.5 Hz), 7.46 (2 H, d, J = 15.8 Hz), 8.7−9.9 (4 H, br s, D_2O exchangeable); EIMS m/z 340 ($M^{\bullet+}$, 5.0). Anal. ($C_{19}H_{16}O_6$) C, H.

1-(3,4-Dihydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (7): yellow powder from $CHCl_3$; mp 165−167 °C; ^{1}H NMR (DMSO-d_6) δ 3.84 (3 H, s), 6.06 (1 H, s), 6.58 (1 H, d, J = 15.8 Hz), 6.75 (1 H, d, J = 15.8 Hz), 6.79 (1 H, d, J = 8.2 Hz), 6.82 (1 H, d, J = 8.2 Hz), 7.02 (1 H, dd, J = 8.2, 1.8 Hz), 7.09 (1 H, d, J = 1.8 Hz), 7.14 (1 H, dd, J = 8.2, 1.8 Hz), 7.32 (1 H, d, J = 1.8 Hz), 7.47 (1 H, d, J = 15.8 Hz), 7.54 (1 H, d, J = 15.8 Hz), 9.19 (1 H, br s, D_2O exchangeable), 9.60−9.70 (2 H, br s, D_2O exchangeable); HRMS m/z calcd for $C_{20}H_{18}O_6$ ($M^{\bullet+}$) 354.1103, obsd 354.1101. Anal. ($C_{20}H_{18}O_6$) C, H.

Hydrogenation of Curcumin (5). Tetrahydrocurcumin (8). Compound **5** (1.1 g, 0.003 mol) in EtOAc (50 mL) was hydrogenated over 10% Pd/C (100 mg) for 2 h. The catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was crystallized from ether/hexane to give 0.9 g (81%) of **8** as colorless crystals: mp 94−95 °C (lit [11] mp 95−96 °C).

Preparation of Radiolabeled DNA Substrates. The following oligonucleotides were HPLC purified by and purchased from Midland Certified Reagent Co. (Midland, TX): AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC-3′; AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE157, 5′-GAAAGCGACCGCGCC-3′, AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′. To analyze the extents of 3′-processing or strand transfer using 5′-end labeled substrates, AE118 was 5′-end-labeled using T_4 polynucleotide kinase (Gibco BRL) and [γ-^{32}P]ATP (DuPont-NEN). The kinase was heat-inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label.

To analyze the extent of strand transfer using the "precleaved" substrate, AE118S was 5′-end-labeled, annealed to AE117, and column purified as above. To determine the extent of 30-mer target strand generation during disintegration, AE156 was 5′-end-labeled, annealed to AE157, AE146, and AE117, and column purified as above.

Integrase Proteins and Assays. Recombinant HIV-1 integrase was purified as described.[23] Integrase at a final concentration of 200 nM was preincubated with inhibitor for 30 min at 30 °C in reaction buffer (50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (wt/vol), 7.5 mM $MnCl_2$, 0.1 mg/mL bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethyl sulfoxide, and 25 mM MOPS, pH 7.2). Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was added, and the reaction continued for an additional 1 h. The final reaction volume was 16 μL.

The disintegration reactions were performed as above with the "Y"-branched oligonucleotide substrate and an integrase deletion mutant, IN^{50-212}, which lacks the N-terminal zinc finger and C-terminal DNA-binding domains.

Electrophoresis and Quantitation. Reactions were quenched by the addition of an equal volume (16 μL) of Maxam−Gilbert loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromophenol blue). An aliquot (5 μL) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate, pH 8.3, 2 mM EDTA, 20% acrylamide, and 8 M urea). Gels were dried, exposed in a Molecular Dynamics PhosphorImager cassette, and analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Percent inhibition was calculated using the following equation:

$$\%I = 100 \times [1 - (D - C)/(N - C)]$$

where C, N, and D are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus integrase, and integrase plus drug, respectively. IC_{50} was determined by plotting the drug concentration versus percent inhibition and determining the concentration which produced 50% inhibition.

DNA Binding via Schiff Base Formation. This procedure was performed essentially as described previously.[6] Integrase (200 nM) was preincubated with the inhibitor for 30 min at 30 °C in reaction buffer (see above). Then, the viral DNA substrate (20 nM) containing a single enzymatically generated abasic site was added for 2 min at room temperature. Sodium borohydride was added for an additional 5 min. An equal volume (16 μL) of 2× SDS−PAGE buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added to each reaction mixture, and the reaction mixture was heated at 95 °C for 3 min prior

to loading a 20 μL aliquot on a 12% SDS−polyacrylamide gel. The gel was run at 120 V for 1.5 h, dried, and exposed in a PhosphorImager cassette.

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