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Short Communication

INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 INTEGRASE BY CURCUMIN

ABHIJIT MAZUMDER, KRISHNAMACHARI RAGHAVAN, JOHN WEINSTEIN, KURT W. KOHN and YVES POMMIER*

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892-4255, U.S.A.

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Abstract—Curcumin (diferuloylmethane) is the yellow pigment in turmeric (*Curcuma longa* L.) that is widely used as a spice, food coloring (curry) and preservative. Curcumin exhibits a variety of pharmacological effects including antitumor, anti-inflammatory, and anti-infectious activities and is currently in clinical trials for AIDS patients. The effects of curcumin have been determined on purified human immunodeficiency virus type 1 (HIV-1) integrase. Curcumin has an inhibitory concentration$_{50}$ (IC_{50}) for strand transfer of 40 μM. Inhibition of an integrase deletion mutant containing only amino acids 50-212 suggests that curcumin interacts with the integrase catalytic core. Two structural analogs, methyl cinnamate and chlorogenic acid, were inactive. Energy minimization studies suggest that the anti-integrase activity of curcumin could be due to an intramolecular stacking of two phenyl rings that brings the hydroxyl groups into close proximity. The present data suggest that HIV-1 integrase inhibition may contribute to the antiviral activity of curcumin. These observations suggest new strategies for antiviral drug development that could be based upon curcumin as a lead compound for the development of inhibitors of HIV-1 integrase.

Key words: curcumin; human immunodeficiency; virus Type-1; integrase; inhibition; antiviral; structure–activity

Several enzymatic steps in the replication cycle of HIV† can be targeted for chemotherapeutic intervention, most notably, reverse transcription and proteolytic cleavage [1]. Research is now in progress to develop clinically active agents against other steps in the viral life cycle. Towards this goal, several laboratories have investigated the pharmacological activity of various drugs as inhibitors of HIV integrase [2–5].

Retroviruses encode the integrase protein at the 3′-end of the *pol* gene. This enzyme, a proteolytic cleavage product of a *gag-pol* fusion protein precursor, is contained in the virus particle and is required for viral replication [6]. It integrates a double-stranded DNA copy of the RNA genome, synthesized by reverse transcriptase, into a host chromosome. During viral infection, integrase catalyzes the excision of the last two nucleotides from each 3′-end of the linear viral DNA, leaving the terminal dinucleotide CA-3′-OH at these recessed 3′-ends. This activity is referred to as the 3′-processing or dinucleotide cleavage. After transport to the nucleus as a nucleoprotein complex, integrase catalyzes a DNA strand transfer reaction involving the nucleophilic attack of these ends on the host chromosome. For recent reviews, see Katz and Skalka [1], Vink and Plasterk [7], and Goff [8].

Curcumin (diferuloylmethane) is the coloring dye in the Indian spice turmeric that is obtained as the powdered root of the rhizome of *Curcuma longa* L. [9]. Turmeric is used as a spice and food colorant in curry and has been used for its various medicinal properties in folk medicine [9]. Curcumin exhibits anti-inflammatory [9, 10] and gastrointestinal activity, possibly as a result of its free radical scavenging properties [11] and inhibitory activity against a variety of protein kinases including protein kinase C [12] and phosphorylase kinase [13]. Curcumin also inhibits carcinogenesis and cancer growth [14–16]. More recently, curcumin has been shown to inhibit HIV replication [17]. The pharmacological safety of curcumin, demonstrated by its consumption for centuries by people in certain countries [9], makes curcumin a potentially interesting compound for investigation. The structure of curcumin is shown in Fig. 1. Due to its structural similarity with CAPE and flavones, which have been found previously to have inhibitory activity *in vitro* against integrase [5], we decided to test curcumin in three integrase assays.

The present study demonstrates that curcumin inhibits the HIV-1 integrase protein, indicating that integrase inhibition may contribute to the anti-HIV activity of curcumin [17]. We also found that an HIV-1 integrase mutant (IN^{50-212}) lacking the N-terminal zinc finger (first 49 amino acids) and the C-terminal DNA-binding domain (last 66 amino acids), which cannot catalyze the 3′-processing and strand transfer reactions [18] but can perform the disintegration [19] reaction, was also inhibited by curcumin. These results demonstrate that curcumin is an HIV-1 integrase inhibitor and suggest that curcumin analogs may be developed as anti-AIDS drugs.

Materials and Methods

Preparation of radiolabeled DNA substrates. The following oligonucleotides were purchased from the Midland Certified Reagent Company (Midland, TX);

* Corresponding author: Tel. (301) 496-5944; FAX (301) 402-0752.

† Abbreviations: HIV, human immunodeficiency virus; CAPE, caffeic acid phenethyl ester; LTR, long terminal repeat; MOPS, 4-morpholinepropanesulfonic acid; and PMA, phorbol 12-myristate 13-acetate.

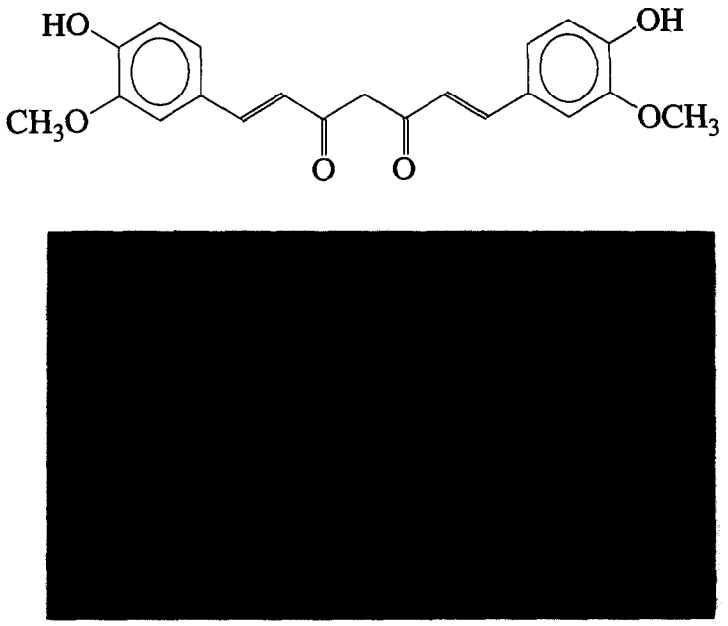

Fig. 1. Chemical structure of curcumin (top) and one of its possible energy minimized structures (bottom). The phenyl rings at either end of the molecule are in a stacked configuration.

AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCG-CTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAG-CAGGGGCTATGGCGTCC-3′; AE117, 5′-ACTGCTA-GAGATTTTCCACAC-3′; AE157, 5′-GAAAGCGA-CCGCGCC-3′; and AE118S, 5′-GTGTGGAAAA-TCTCTAGCA-3′. These oligonucleotides were purified by HPLC. The AE117 and AE118 oligonucleotides correspond to the U5 end of the HIV LTR [20]. For the 3′-processing and strand transfer assay, AE118 was 5′-end-labeled using polynucleotide T_4 kinase and [γ-^{32}P]ATP. The kinase was heat-inactivated, and an equimolar amount of AE117 was added. The mixture was heated at 95° and allowed to cool slowly to room temperature. The reaction was then run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label. For strand transfer reactions using the precleaved oligo, AE118S was labeled, annealed, and purified as above.

3′Processing and strand transfer assays. Purified recombinant HIV-1 integrase wild-type was a gift of Dr. R. Craigie, Laboratory of Molecular Biology, NIDDK. The enzyme was preincubated at a final concentration of 0.25 μM at 30° with curcumin (Sigma) or an analog in reaction buffer (50 mM NaCl, 1 mM HEPES, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 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). After 30 min, 0.3 pmol of the labeled cleavage/strand transfer substrate was added, and the incubation was continued for an additional 60 min at 30°. The final reaction volume was 16 μL. The reaction was quenched by the addition of an equal volume of Maxam-Gilbert loading dye.

Disintegration assays. The disintegration reactions [19] were performed as above with the "Y" oligonucleotide substrate and an integrase deletion mutant, $IN^{50\text{-}212}$, which lacks the N-terminal zinc finger and C-terminal DNA-binding domains [18]. For this assay, AE157 was 5′-end-labeled as above. Equimolar amounts of AE117, AE156, and AE146 were added. The mixture was annealed and run on a G-25 Sephadex quick spin column as above.

Gel electrophoresis and quantitation. An aliquot from each reaction was electrophoresed on a denaturing 20% polyacrylamide gel. Gels were dried and subjected to autoradiography using Kodak XAR-2 film or exposed in a Molecular Dynamics Phosphorimager cassette. Gels were analyzed using a Betascope 603 blot analyzer (Betagen, Waltham, MA). Percent inhibition was calculated as described previously [4].

Results and Discussion

The 3′-processing (dinucleotide cleavage) and strand transfer activities of integrase can be quantitated using a 21-mer double-stranded oligonucleotide, which represents the U5 end of the HIV LTR as the substrate [5, 20]. 3′-Processing liberates a GT dinucleotide from the 3′-end, resulting in the formation of a 19-mer oligonucleotide. Then integrase catalyzes a DNA strand transfer reaction whereby the integrase-processed U5 oligonucleotide with the recessed 3′-end is integrated into another U5 oligonucleotide, which serves as the target DNA. To study inhibition of the strand transfer reaction by itself, a 19-mer oligonucleotide is annealed to a 21-mer oligonucleotide. This 3′-recessed substrate mimics the 3′-processing product and undergoes the strand transfer reaction only. The reverse or "disintegration" activity [19] 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 30-mer. A significant difference between the three assays is that the disintegration assay and the effects of drugs in this assay can be used with integrase deletion mutants since these mutants can still catalyze disintegration but not 3′-processing and strand transfer [18]. Therefore, the disintegration assay with the 50-212 HIV-1 integrase deletion mutant can be used to determine whether drugs act on the enzyme catalytic core [5].

Inhibition of HIV-1 integrase by curcumin. The effects

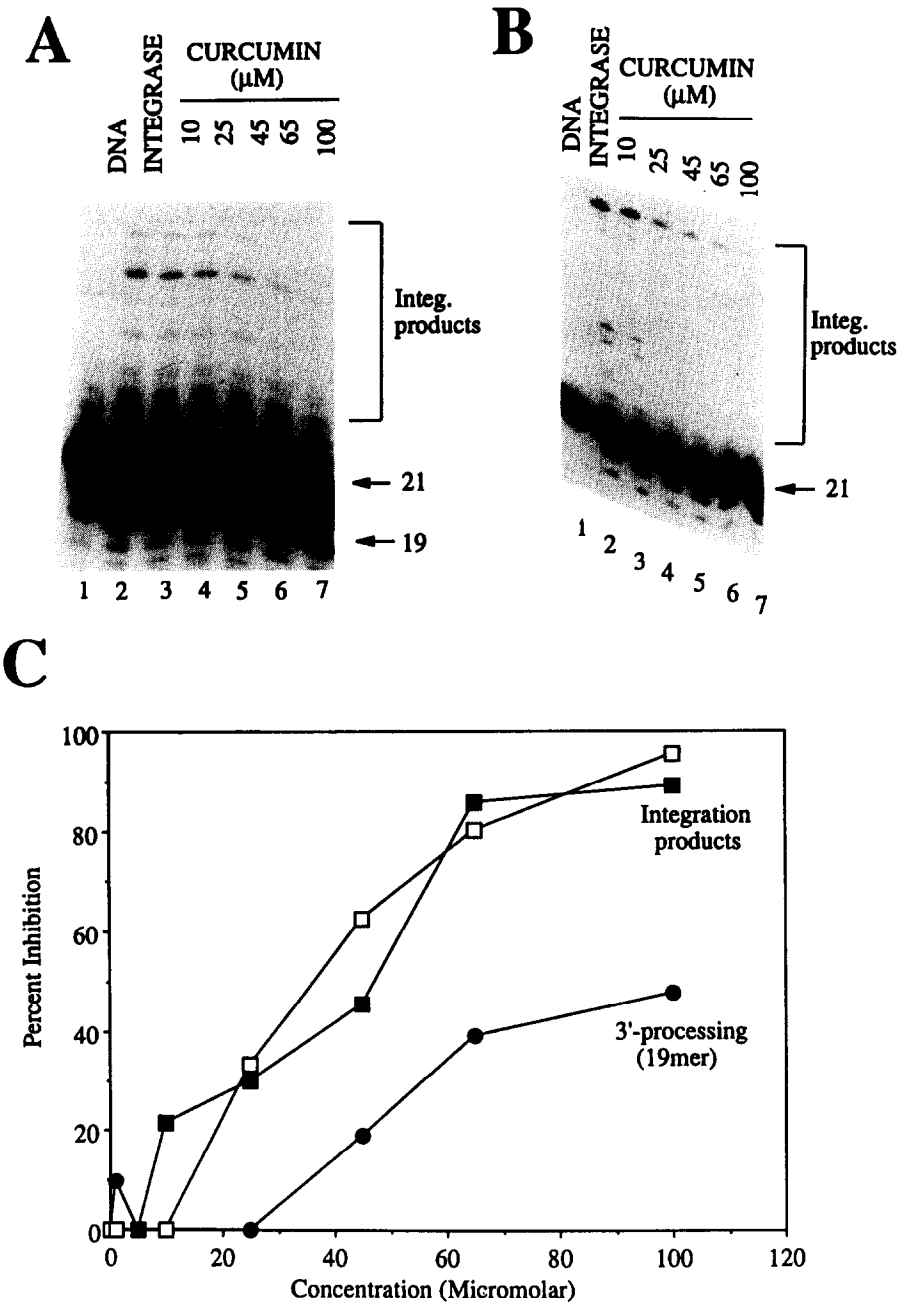

Fig. 2. Inhibition of HIV-1 integrase-catalyzed 3′-processing and strand transfer by curcumin. (A) Phosphorimager picture showing 3′-processing and strand transfer reactions in the presence of curcumin. Lane 1, labeled DNA alone; lane 2, integrase without drug; lanes 3–7, integrase in the presence of the indicated concentrations of curcumin. The DNA strand transfer (integration) products are indicated by the bracket. The 3′-processing product (19-mer, indicated by the lower arrow) and the DNA substrate (21-mer, indicated by the upper arrow) are also shown. The band corresponding to the substrate 21-mer (and also, therefore, the contaminating shorter oligonucleotides) has been overexposed in order to show the 3′-processing and strand transfer products more readily. (B) Phosphorimager picture showing inhibition of strand transfer using the precleaved oligonucleotide (19-mer) in the presence of increasing concentrations of curcumin. Lane 1, labeled DNA alone; lane 2, integrase without drug; lanes 3–7, integrase in the presence of the indicated concentrations of curcumin. (C) Concentration–response curves showing the percent inhibition of each reaction in the presence of increasing concentrations of curcumin. The data from a typical experiment, shown in panels A and B, were quantitated to obtain the graph. Inhibition of DNA strand transfer (integration) using the blunt-ended or precleaved oligonucleotide and of 3′-processing is depicted by the filled squares, open squares, and filled circles, respectively.

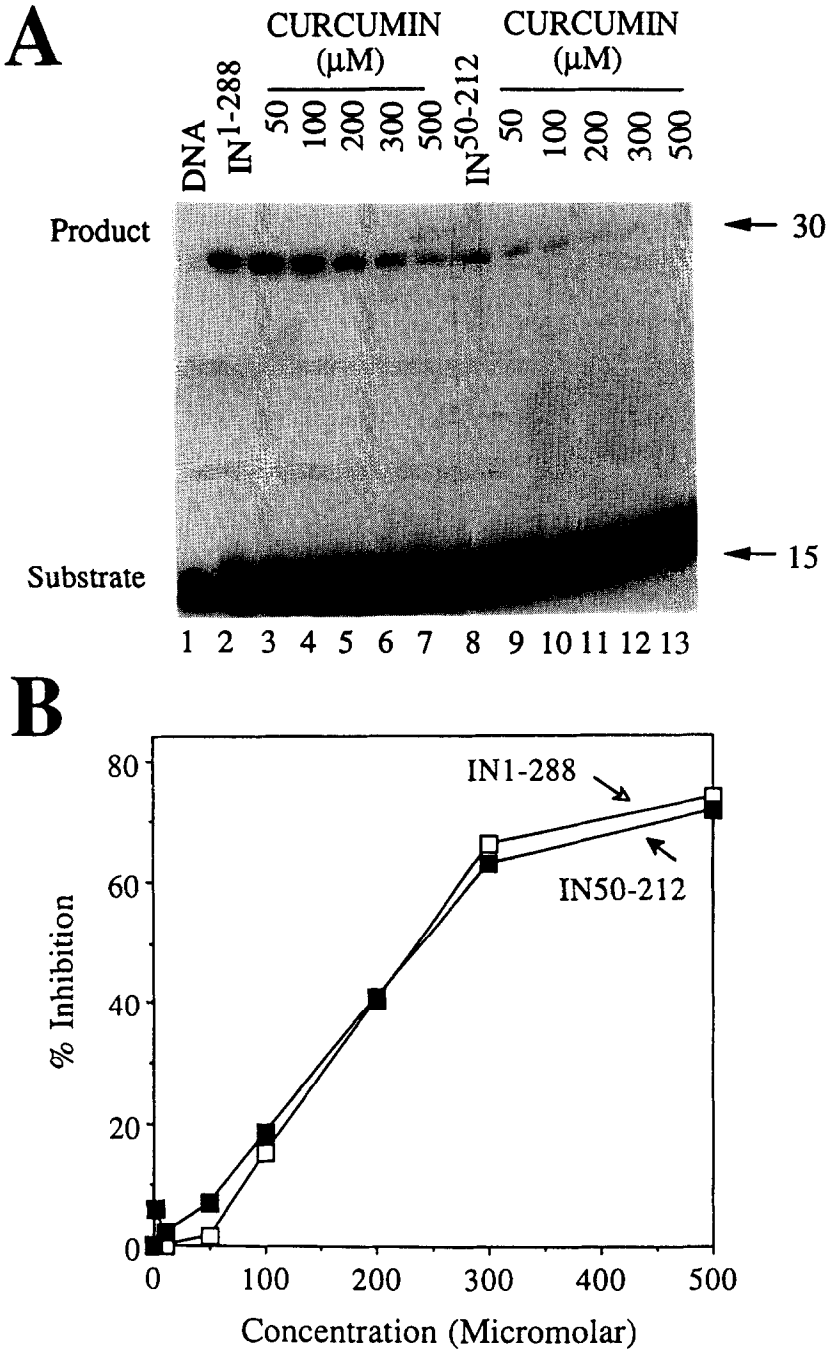

Fig. 3. Effect of curcumin upon disintegration catalyzed by the full size HIV-1 integrase (IN^{1-288}) and the deletion mutant IN^{50-212}. (A) Phosphorimager picture showing disintegration reactions. Lane 1, DNA alone; lane 2, IN^{1-288} without drug; lanes 3–7, disintegration obtained using the IN^{1-288} in the presence of the indicated concentrations of curcumin; lane 8, IN^{50-212} without drug; lanes 9–13, disintegration obtained using the IN^{50-212} in the presence of the indicated concentrations of curcumin. (B) Concentration–response curves showing the percent inhibition of each disintegration in the presence of increasing concentrations of curcumin. The data from a typical experiment, shown in panel A, were quantitated to obtain the graph. The inhibition of disintegration catalyzed by the full size HIV-1 integrase (IN^{1-288}) and by the deletion mutant IN^{50-212} is depicted by the open and filled squares, respectively.

of curcumin and its analogs (chlorogenic acid and methyl cinnamate) on 3′-processing (dinucleotide cleavage) and strand transfer catalyzed by HIV-1 integrase were examined. Curcumin inhibited both 3′-processing and strand transfer with IC_{50} values of approximately 95 and 40 μM, respectively (Fig. 2). The variability between experiments was ± 20%. In contrast, the analogs did not inhibit integrase at concentrations up to 100 μM (data not shown). Therefore, curcumin, which selectively inhibits HIV-1 production of p24 antigen with an IC_{50} of 4 μM [17], inhibited strand transfer catalyzed by HIV-1 integrase *in vitro* at a concentration one order of magnitude higher. This difference however, could be due to the intracellular concentration of curcumin.

Action of curcumin on the central catalytic domain of the integrase. In an attempt to define the curcumin binding site on HIV-1 integrase in more detail, an integrase deletion mutant (IN^{50-212}) lacking the N-terminal zinc-finger region and the C-terminal DNA-binding domain [18] was assayed for inhibition by curcumin. This mutant could only be used in the disintegration assay because it has been well established that both the amino- (zinc finger) and carboxy- (DNA-binding) terminal domains are required for the HIV-1 3′-processing and strand transfer reactions [18]. Curcumin inhibited the disintegration mediated both by the wild-type (IN^{1-288}) and the truncated mutant (IN^{50-212}) enzymes at similar drug concentrations (Fig. 3). As demonstrated previously [18], disintegration efficiency was reduced markedly by a factor of three to four with the IN^{50-212} enzyme. The concentration of curcumin required for inhibition of disintegration was higher than that required for inhibition of either 3′-processing or strand transfer. These results are consistent with those observed with other molecules [5]. This observation suggests that the active site of HIV-1 integrase may be more tolerable of drug-induced protein or DNA distortion during the disintegration reaction, consistent with the relative tolerance of integrase to mutagenesis of either substrate features [21] or protein structural domains [18] in this reaction. The finding that curcumin was active against the IN^{50-212} mutant (Fig. 3) implies that the binding of curcumin to the integrase core region is probably responsible for integrase inhibition. Binding of curcumin to DNA has never been reported. It seems unlikely because the curcumin structure does not resemble that of a typical DNA groove binder or intercalator.

Structure–activity relationships and binding site of curcumin in the HIV-1 integrase. It is interesting that curcumin is active against HIV-1 integrase in light of the fact that the structurally related compounds chlorogenic acid and methyl cinnamate demonstrated no inhibition against either 3′-processing or strand transfer up to 100 μM (data not shown). These results suggest the possibility that the presence of ortho phenolic hydroxyl groups [5] may not be the only structural characteristic that results in activity against the integrase. Second, the possibility of a covalent adduct between a nucleophile on the integrase and curcumin does not seem likely given that each of the three compounds can undergo a Michael addition and yet only one of these compounds, curcumin, was able to inhibit the enzyme. Further evidence arguing against the formation of a covalent adduct is the finding that a methyl cinnamate analog of CAPE [4, 5], which does not contain a double bond and, therefore, would not be able to undergo a Michael addition, still inhibited the integrase (Mazumder A and Pommier Y, unpublished observations).

Energy minimization studies showed that curcumin is able to fold back on itself such that the phenyl rings can stack on each other (Fig. 1). Therefore, such stacking interactions with the hydroxyl groups from each phenyl ring in close proximity at the integrase active site may be important determinants in inhibitor potency. The same conclusion was reached by examination of the structure–activity relationship within the flavone family of HIV-1 integrase inhibitors [5]. Inhibition by flavones usually required the presence of at least one ortho pair of phenolic hydroxyl groups. However, an exception to this observation was provided by NSC 339192. This molecule has only two hydroxyl groups, and these are not in ortho configuration, but rather are on different rings. Computer modeling suggested that this molecule could also fold such that the phenolic groups stack above each other [5].

Site-directed mutagenesis and sequence alignment have identified three amino acid residues in the catalytic core that are conserved among all retroviral integrases [22] and are critical for activity [23, 24]. These are Asp 64, Asp 116 and Glu 152. Such acidic residues are known to be essential for the 3′-5′ exonuclease activity of the Klenow fragment of DNA polymerase I due to their coordination to metal ions and the phosphodiester backbone at the active site [25]. Therefore, inhibition by curcumin may result from its binding to the catalytic core in a manner that facilitates coordination of its hydroxyl groups to the acidic residues in the integrase active site, resulting in a loss of interaction of these same residues with the DNA substrate or metal cofactor. Inhibition of other enzymes such as the Klenow fragment of DNA polymerase by curcumin has not been reported. Therefore, the mechanism of inhibition outlined above awaits further investigation.

Relevance to antiviral drug mechanisms. Previous work has shown that curcumin inhibits p24 antigen production and *Tat*-mediated transcription [17]. Our results demonstrating that curcumin also inhibits purified HIV-1 integrase suggest that the anti-HIV activity of curcumin, currently in clinical trials for AIDS patients, could be due to several mechanisms. The relative lack of selectivity of curcumin is exemplified by the inhibitory activity of curcumin against PMA-responsive elements (TRE) binding to its DNA recognition site [15] and the anti-protein kinase activities of curcumin [12, 13]. Although the inhibition of integrase by curcumin may not be the only mechanism by which it exerts its antiviral effects, this inhibition may contribute to the potency of curcumin against HIV-1 replication. Nevertheless, curcumin may be a lead compound to develop analogs of greater potency against HIV-1 integrase that would exhibit clear antiviral activity.

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