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Inhibitors of human immunodeficiency virus integrase

(retrovirus/AIDS/topoisomerase/zinc finger)

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ABSTRACT **In an effort to further extend the number of targets for development of antiretroviral agents, we have used an *in vitro* integrase assay to investigate a variety of chemicals, including topoisomerase inhibitors, antimalarial agents, DNA binders, naphthoquinones, the flavone quercetin, and caffeic acid phenethyl ester as potential human immunodeficiency virus type 1 integrase inhibitors. Our results show that although several topoisomerase inhibitors—including doxorubicin, mitoxantrone, ellipticines, and quercetin—are potent integrase inhibitors, other topoisomerase inhibitors—such as amsacrine, etoposide, teniposide, and camptothecin—are inactive. Other intercalators, such as chloroquine and the bifunctional intercalator ditercalinium, are also active. However, DNA binding does not correlate closely with integrase inhibition. The intercalator 9-aminoacridine and the polyamine DNA minor-groove binders spermine, spermidine, and distamycin have no effect, whereas the non-DNA binders primaquine, 5,8-dihydroxy-1,4-naphthoquinone, and caffeic acid phenethyl ester inhibit the integrase. Caffeic acid phenethyl ester was the only compound that inhibited the integration step to a substantially greater degree than the initial cleavage step of the enzyme. A model of 5,8-dihydroxy-1,4-naphthoquinone interaction with the zinc finger region of the retroviral integrase protein is proposed.**

Although human immunodeficiency virus (HIV) integrase-mediated integration of HIV DNA into the host genome is essential to the virus life cycle, to date pharmacologic antiretroviral research has neglected this enzyme, focusing principally on agents that inhibit other virally encoded enzymes, such as HIV reverse transcriptase or HIV protease (1). *In vitro* systems using oligonucleotide substrates and purified integrase protein have been invaluable for investigating the DNA substrate requirements and chemical mechanisms of the reactions catalyzed by the HIV integrase (2–11). The development of an *in vitro* assay of integrase function now permits rapid testing of a large number of compounds as potential inhibitors of the HIV integrase. We report here an investigation of the effects of many such inhibitors on the cleavage and strand-transfer reactions; to our knowledge the only previous study is the demonstration (12) that aurintricarboxylic acid and its relatives inhibit integrase-promoted cleavage. Development of a clinically tolerable inhibitor of the HIV integrase could have profound implications for antiretroviral therapy, including potential synergy with currently available reverse transcriptase inhibitors, as well as prevention of a chronic carrier state.

MATERIALS AND METHODS

Materials. HIV-1 integrase protein (3.5 pmol per reaction), produced via an *Escherichia coli* expression vector as described (13), was obtained from R. Craigie (Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases) and stored at −70°C in 1 M NaCl/20 mM Hepes, pH 7.6/1 mM EDTA/1 mM dithiothreitol/20% glycerol (wt/vol). Caffeic acid phenethyl ester (CAPE) was brought to our attention by Dezider Grunberger (Columbia University, New York), who supplied the compound. Doxorubicin, 5-iminodaunorubicin, mitoxantrone, ellipticine, ellipticinium, 9-aminoacridine, amsacrine, ditercalinium, ethidium, camptothecin, 9-aminocamptothecin, 10,11-methylenedioxycamptothecin, etoposide (VP-16), teniposide (VM-26), and quercetin were obtained through the Developmental Therapeutics Program, National Cancer Institute. Hydroxyrubicin and adriamycinone were obtained through Waldemar Priebe (M. D. Anderson Hospital, Houston). Naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, and dihydroxyanthraquinone were purchased from Aldrich. Chloroquine, primaquine, quinacrine, and amodiaquine were obtained through Sigma. Hydroxychloroquine was from Sterling–Winthrop Research Institute. Mefloquine was from Hoffmann–La Roche.

Oligonucleotide Substrate. Oligonucleotides were obtained from Midland Certified Reagent (Midland, TX), and were HPLC-purified before use. The following complementary oligonucleotides were used as substrates:

AE118: 5′-GTGTGGAAAATCTCTAGCAGT-3′ and

AE117: 5′-ACTGCTAGAGATTTTCCACAC-3′ (2).

AE118 (1 μl of 0.1 mg/ml) was 5′-radiolabeled by treating it with [^{32}P]ATP and polynucleotide kinase at 37°C for 45 min followed by 15 min at 85°C to inactivate the kinase. The oligonucleotide was subsequently slowly hybridized by mixing with 1 μl of AE117 at 0.4 mg/ml. Unincorporated nucleotides were separated from labeled oligonucleotide by passage through a G-25 quick spin column (Boehringer Mannheim).

HIV Integrase Assay. The stock enzyme (0.44 mg/ml) was first diluted 1:3 in protein storage buffer 1 M NaCl/20 mM Hepes, pH 7.6/1 mM EDTA/1 mM dithiothreitol/20% (wt/vol) glycerol. Subsequent enzyme dilution was at 1:20 in reaction buffer (25 mM Mops, pH 7.2/7.5 mM $MnCl_2$/bovine serum albumin at 100 μg/ml/10 mM 2-mercaptoethanol) to give 50 mM NaCl/1 mM Hepes/50 μM EDTA/50 μM dithiothreitol/10% (wt/vol) glycerol/7.5 mM $MnCl_2$/bovine serum albumin at 0.1 mg/ml/10 mM 2-mercaptoethanol/25 mM Mops, pH 7.2. Reaction volume was 16 μl. All reactions were done in 10% dimethyl sulfoxide to enhance the reaction efficiency and as a universal drug solvent. Reactions were for 1 hr with 0.3 pmol of ^{32}P-labeled double-stranded oligonucleotide. Reactions were stopped by adding 16 μl of Maxam–

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Abbreviations: DHNQ, dihydroxynaphthoquinone; CAPE, caffeic acid phenethyl ester; top2, topoisomerase II; HIV, human immunodeficiency virus.

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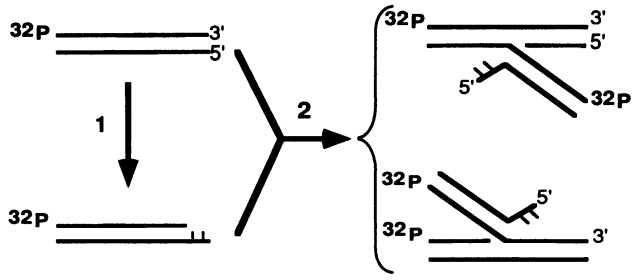

FIG. 1. Sequential cleavage and integration reactions in the retroviral integrase assay. The cleavage reaction removes a dinucleotide from the 3′ end of one of the strands at the integration site, thereby converting the ^{32}P-labeled 21-mer to a 19-mer (step 1). Integration (step 2) can occur at several sites in either recipient strand.

Gilbert loading buffer to each 16-μl sample. Subsequently 4 μl of each sample was run on a 20% denaturing polyacrylamide gel in 1× TBE buffer (TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3). Gels were dried, and autoradiography was done by using Kodak XAR-2 film.

Quantitation. Dried gels were analyzed by using a Betascope 603 blot analyzer (Betagen, Waltham, MA). Radioactivity was counted in the 19-mer cleavage band, the larger integration bands (to avoid interference from the 21-mer origin band) and the lane total. Percent inhibition was calculated by the following equation:

$$100 \times [1 - (D - C)/(N - C)],$$

where C, N, and D are the fractions of 21-mer converted to 19-mer or integration products for DNA alone, DNA plus integrase, and DNA plus integrase plus drug, respectively. IC_{50} is the concentration of compound producing 50% inhibition. IC_{50} values were calculated from a sigmoid model by using the formula:

$$y = [100\ x^n]/[(IC_{50})^n + x^n],$$

where x is the concentration of compound tested, y is percent inhibition, and n is the Hill coefficient and was set at 1.2, a mean.

RESULTS AND DISCUSSION

Fig. 1 describes the *in vitro* integrase assay. Purified HIV integrase is treated with a 21-mer oligonucleotide corresponding to the U5 end of the HIV proviral DNA. The initial step involves nucleolytic cleavage of two bases from the 3′ end. Subsequently, these recessed 3′ ends are joined, through a strand-transfer reaction, to the 5′ end of an integrase-induced break in an identical second oligonucleotide, which serves as the target DNA. Reaction products were separated by electrophoresis on a 20% denaturing acrylamide gel. A β-emission detector was used to quantitate dried gels. Results were expressed as percentage inhibition of the nucleolytic cleavage and integration products. Typical results for HIV integrase in the absence of inhibitor were 12–15% nucleolytic cleavage and 2–5% strand-transfer/integration products in a reaction where the molar ratio of integrase to DNA was 10:1.

Our initial goal, to test whether DNA topoisomerase inhibition correlated with integrase inhibition, was prompted by reports of antiretroviral activity of camptothecins (14), as well as by analogies between the mechanisms of the integrase and topoisomerase II (top2). These analogies include the production of 5′ overhangs in DNA, the ability to recombine cleaved double-stranded DNA, and the possibility of a covalently linked intermediate (15, 16).

Doxorubicin (compound **1**), a potent top2 inhibitor and a clinically important antitumor agent, was among the most potent inhibitors of HIV integrase-mediated DNA cleavage and integration tested (Figs. 2–5, Table 1). Several analogs of doxorubicin were analyzed to evaluate the importance of DNA binding. As this binding is stabilized by the cationic charge of the sugar amino group, replacement of this amino group with a hydroxy group, as in hydroxyrubicin (compound **2**), would be expected to reduce DNA binding (Table 1). Hydroxyrubicin inhibited the integrase with 12- to 16-fold less potency than did doxorubicin (Figs. 2–5, Table 1). However, the inhibition by hydroxyrubicin was similar to that produced by 5-iminodaunorubicin (compound **3**) (Figs. 2–5, Table 1) which retains the sugar amino group and binds to DNA with affinity similar to doxorubicin. Doxorubicin, hydroxyrubicin, and 5-iminodaunorubicin all inhibit top2 and possess antitumor activity. Doxorubicin aglycone (compound **4**), which lacks the sugar moiety, was found by DNA-supercoil-relaxation assays to bind DNA very weakly and inhibited HIV integrase cleavage detectably only at 100 μM (39% inhibition of cleavage) (Figs. 2–5, Table 1).

Several other types of DNA-intercalating top2 inhibitors were effective inhibitors of HIV integrase, including mitoxantrone (compound **5**), ellipticines, and their related derivative intoplicine (RP-60475, compounds **10–12**) (Figs. 2–5, Table 1). However, amsacrine, another DNA-intercalating top2 inhibitor, had no detectable effect on the HIV integrase. Quercetin, which is a weak DNA intercalator and a weak top2 inhibitor, was a potent HIV integrase inhibitor (Table 1) (31).

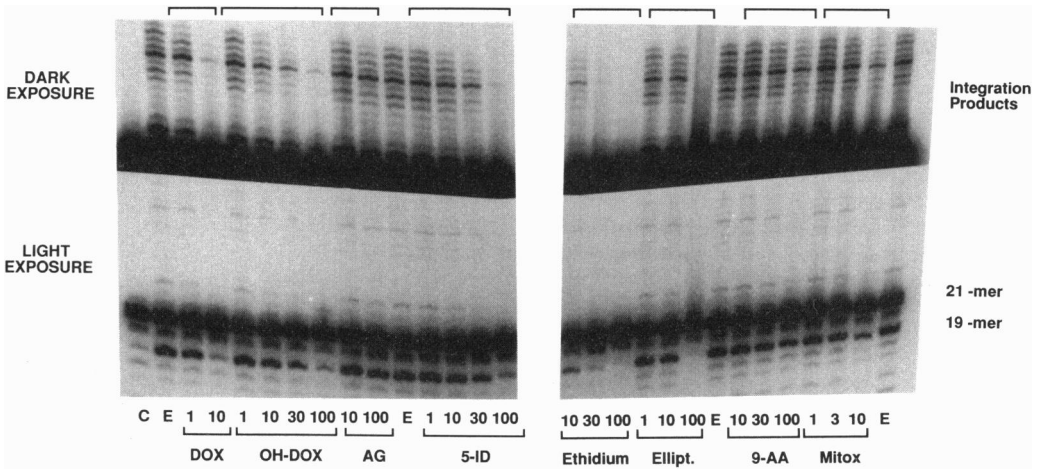

FIG. 2. HIV integrase assay. C, DNA control; E, enzyme alone; DOX, doxorubicin; OH-DOX, hydroxyrubicin; AG, adriamycinone (doxorubicin aglycone); 5-ID, 5-iminodaunorubicin; Ellipt., ellipticine; 9-AA, 9-aminoacridine; Mitox, mitoxantrone.

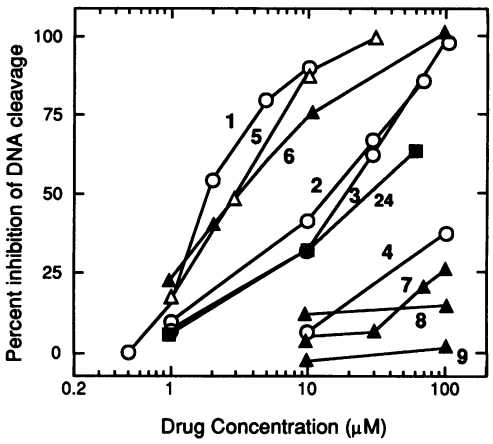

FIG. 3. Inhibition of the nucleolytic cleavage reaction of HIV integrase by anthracyclines related to doxorubicin (compounds **1–4**, ○), mitoxantrone (compound **5**, △), naphthoquinones (compounds **6–9**, ▲), and quercetin (compound **24**, ■). Compounds are numbered as in Table 1, and structures are shown in Fig. 5.

In addition, both of the epipodophyllotoxins, etoposide (VP-16) and teniposide (VM-26), which do not bind DNA detectably, but block top2 complexes, possibly by a base-stacking mechanism (32), were inactive against the integrase. The DNA topoisomerase I inhibitor camptothecin and its two more potent analogs were also inactive in the integrase assay (Table 1). These drugs were also unable to prevent acute infection of ATH8 cells by HIV-1 when used 1 hr after initial inoculation at concentrations up to 100 μM (H. Mitsuya, personal communication). Topoisomerase inhibition, therefore, does not appear to strongly correlate with integrase inhibition. This result is consistent with recent data by Craigie and coworkers (33), which show that the integrase reaction proceeds without the presence of a covalent DNA-enzyme intermediate.

Because our results demonstrated a lack of correlation between inhibition of top2 and inhibition of integrase, we expanded the compounds screened to include a broad spectrum of DNA-binding affinities and properties to ascertain whether integrase inhibition correlated with DNA binding. Along with doxorubicin, another compound with a high DNA-binding affinity, ditercalinium, was found to be a very potent cleavage and integration inhibitor (Table 1, Fig. 4). Chloroquine, a weak DNA intercalator used to treat malaria, was tested in the hope of identifying an HIV-integrase inhibitor devoid of the clinical toxicity of strong DNA intercalators. Among the antimalarials tested, chloroquine was active against the integrase (IC_{50} = 13 μM), whereas

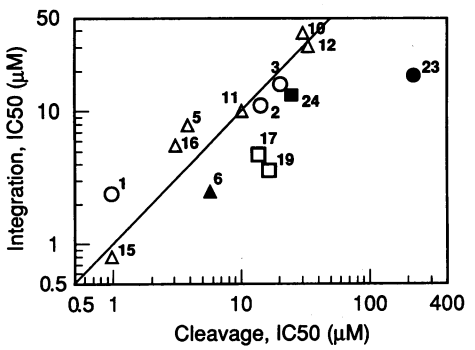

FIG. 4. Inhibition of integration relative to inhibition of nucleolytic cleavage. ○, Anthracyclines; △, other DNA intercalators; ▲, dihydroxynaphthaquinone; □, antimalarials; ●, CAPE; ■, quercetin. Numbers correspond to structures in Fig. 5. The diagonal line is theoretical for equivalence between integration and cleavage.

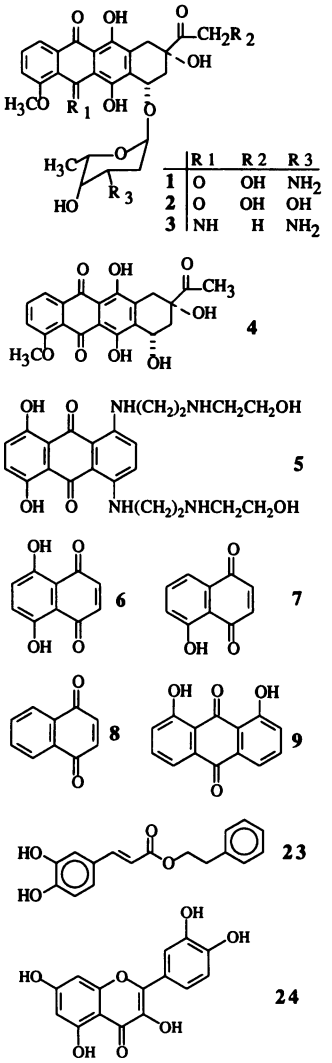

FIG. 5. Structures of several of the compounds tested. Numbers are the same as for Table 1.

quinacrine, a stronger intercalator, was inactive (Table 1). Similarly, other types of DNA binders, including polyamines and minor-groove binders such as spermine, spermidine, and distamycin, did not inhibit the HIV integrase. Also without effect was the DNA intercalator 9-aminoacridine (compound **13**, Fig. 2, Table 1). Primaquine, a chloroquine relative having little or no affinity for DNA, was as effective as chloroquine in the integrase assay. Thus, as with inhibition of top2, there is no strong correlation between DNA-binding affinity of DNA intercalators and enzyme inhibition. The inactivity of certain DNA intercalators may be from a number of confounding factors, including local differences in the mode of intercalation such as minor- or major-groove binding, residence time, or sequence selectivity. Although many of these compounds have been tested for sequence selectivity in various systems, the literature is incomplete and at times contradictory. Thus, as with top2 inhibition, when the correlation between integrase inhibition and DNA binding was evaluated among a broad range of compounds, there were marked discrepancies. Therefore, enzyme inhibition may involve direct interaction of drug with enzyme in addition to DNA intercalation.

Several classes of non-DNA binders were then tested. Substituted 1,4-naphthoquinones were examined to test whether a moiety common to the structures of doxorubicin and mitoxantrone could be a "master key" responsible for

Table 1. HIV integrase inhibition and DNA binding

Compound (abbreviation)	IC_{50}, μM		log K_{app} (M^{-1})	Ref.
	Cleavage†	Integration‡	[Na^+]*	
1 Doxorubicin (DOX)	0.9 ± 0.7	2.4	6.62 [100]	17
			6.46 [185]§	
2 Hydroxyrubicin (HO-DOX)	14.4 ± 2.7	11.3	5.28 [185]§	
3 5-Iminodaunorubicin (5-ID)	20.4 ± 4.3	16.2	¶	18
4 Adriamycinone (AG)	>100	>100	‖	
5 Mitoxantrone (Mitox)	3.85 ± 0.6	8.0	6.81 [100]	19
6 Dihydroxynaphthoquinone (DHNQ)	5.73 ± 2.7	2.5	**	
7 5-Hydroxynaphthoquinone (5-HNQ)	>100	>100	**	
8 Naphthoquinone (NQ)	>100	>100	**	
9 Dihydroxyanthraquinone (DHAQ)	>100	>100	**	
10 Ellipticine	30.1 ± 3.8	39.3	5.11 [100]	20
11 Elliptinium	10.1 ± 2.1	10.3	6.11 [100]	20
12 Intoplicine (RP-60475)	33.4 ± 9.4	31.4	5.34 [200]	21
13 9-Aminoacridine (9-AA)	>100	>100	5.60 [10]	22
14 *m*-AMSA	>100	>100	5.15 [10]	22
			4.30 [100]	23
15 Ditercalinium	0.9 ± 0.6	0.8	7.00 [100]	24
16 Ethidium	3.0 ± 0.9	5.66	5.63 [100]	25
17 Chloroquine	13.1 ± 10	5.14	3.65 [150]	26
18 Hydroxychloroquine	>100	>100		
19 Primaquine	15.3 ± 3.6	3.62	††	
20 Quinacrine	>100	>100	6.08 [5]	27
21 Mefloquine	>100	>100		
22 Amodiaquine	>100	>100		
23 Caffeic acid phenethyl ester (CAPE)	220 ± 42	18.9 ± 10.1	‡‡	
24 Quercetin	19.4 ± 9.9	11.0 ± 5.9	§§	
25 Camptothecin	>100	>100	††	
26 9-NH_2-camptothecin	>100	>100	††	
27 10,11-CH_2O_2-camptothecin	>100	>100	††	
28 Etoposide (VP-16)	>100	>100	††	
29 Teniposide (VM-26)	>100	>100	††	
30 Spermine	>100	>100	6.14 [17]	28
31 Spermidine	>100	>100		
32 Distamycin	>100	>100	6.08 [50]	29

m-AMSA, 4′-(9-acridylamino)methanesulfo-*m*-anisidide.
*K_{app} is the intrinsic DNA-binding constant. Sodium concentration (in brackets) is in mM.
†IC_{50} ± SD (μM) (at least three experiments).
‡IC_{50} (mean of at least two experiments).
§Personal communication, J. Chaires, University of Mississippi Medical Center, Jackson.
¶K_{app} approximately equal to doxorubicin.
‖DNA unwinding 0.5° per bp at 20 μM in simian virus 40.
**No DNA binding detected by unwinding assay (30).
††Little or no DNA binding.
‡‡Chemical structure not suggestive of DNA binding.
§§DNA unwinding 0.4° per bp at 50 μM in pBR322 DNA (16).

activity. Interestingly, 5,8-dihydroxy-1,4-naphthoquinone (DHNQ, compound **6**), a core structure common to these two drugs (Fig. 5), was as active as mitoxantrone (Figs. 3 and 4, Table 1). This compound was further tested by spectroscopic analysis and DNA-unwinding assays (30) and was found not to intercalate DNA. Analogs of DHNQ, compounds **7** and **8**, which lack one or both hydroxyl groups, were inactive, demonstrating the importance of both hydroxyl groups (Fig. 3, Table 1). CAPE (compound **23**, Fig. 5, Table 1), a nontoxic apiary product that selectively inhibits transformed cells (34), was the compound that most clearly demonstrated selective inhibition of the integration step relative to the cleavage step of the integration reaction (Fig. 4, Table 1). The CAPE structure is such that it is unlikely to bind DNA significantly. Other compounds including primaquine, chloroquine, and DHNQ also exhibited selectivity, although to a lesser degree, for inhibition of the integration step. The IC_{50} values for inhibition of integration by these compounds were 2- to 5-fold less than the IC_{50} values for inhibition of the cleavage reaction. The flavonoid quercetin was another compound that was found nearly twice as potent in its inhibition of integration versus cleavage.

Our results demonstrate that a variety of integrase inhibitors can be identified by means of the *in vitro* assay and that two steps of enzyme action, nucleolytic cleavage and strand transfer (integration), can each be evaluated. The relative potencies for inhibition of the cleavage and integration steps by the active compounds are compared in Fig. 4. Although inhibition of integrase is not simply dependent on DNA binding, as some compounds inhibit integrase and yet possess little or no ability to bind DNA, it may be possible to develop inhibitors selectively toxic to integration. The ability of CAPE, and to a lesser extent DHNQ, antimalarials, and quercetin to selectively inhibit the integration step—with an IC_{50} for cleavage an order of magnitude greater than the IC_{50} for integration—suggests that the two steps of the enzyme action can be mechanistically separated by drugs.

The inhibition of HIV integrase by the double-hydroxylated (DHNQ), not the single (5-hydroxynaphthoquinone) or nonhydroxylated naphthoquinone (Fig. 4), suggests the pos-

sibility of DHNQ acting by binding to a zinc finger structure in the HIV integrase. Point-mutation analyses of integrase function by Engleman and Craigie (33, 35, 36) indicate that mutation of the histidines (His-12 and His-16) and cysteines (Cys-40 and Cys-43) of the His-Cys zinc finger region impair cleavage processing and integration while not affecting the reverse or "disintegration" reaction. Thus, this region of the protein appears important for the cleavage and integration steps of the HIV integrase reaction and may be involved in production of a multimeric state of integrase required for both the cleavage and integration steps but not for the disintegration reaction. DHNQ consists of juxtaposed phenolic hydroxy and keto groups that are effective chelators of divalent metal ions. Therefore, DHNQ may displace the imidazole ligands of the zinc finger contributed by the two histidine residues (37). Binding to the His-Cys zinc finger region of the integrase would lead to compromised enzyme activity. If a zinc finger model proves to be correct and essential to integrase function, it may be possible to specifically design compounds that would bind to this region.

Although the *in vitro* assay used in this study has been valuable to the understanding of integrase molecular pharmacology, exploiting this information to develop agents active in preventing HIV replication in intact cells will require development of a simple and safe assay by which the rate of viral integration in intact cells can be measured.

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