Antiretroviral Agents as Inhibitors of both Human Immunodeficiency Virus Type 1 Integrase and Protease

Abhijit Mazumder, Shaomeng Wang,† Nouri Neamati, Marc Nicklaus,† Sanjay Sunder, Julie Chen, George W. A. Milne,† William G. Rice,‡ Terrence R. Burke, Jr.,† and Yves Pommier*

Laboratories of Molecular Pharmacology and Medicinal Chemistry, Division of Basic Sciences, National Cancer Institute, Building 37, Bethesda, Maryland 20892, and Laboratory of Antiviral Drug Mechanisms, NCI−Frederick Cancer Research and Development Center, Frederick, Maryland 21702

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The human immunodeficiency virus type one integrase (HIV-1 integrase) is required for integration of a double-stranded DNA copy of the viral RNA genome into a host chromosome and for HIV replication. We have previously reported that phenolic moieties in compounds such as flavones, caffeic acid phenethyl ester (CAPE), tyrphostins, and curcumin confer inhibitory activity against HIV-1 integrase. We have investigated the actions of several recently described protease inhibitors, possessing novel structural features, on HIV-1 integrase. NSC 158393, which contains four 4-hydroxycoumarin residues, was found to exhibit antiviral, antiprotease, and antiintegrase activity. Both the DNA binding and catalytic activities (3′-processing and strand transfer) of integrase were inhibited at micromolar concentrations. Disintegration catalyzed by an integrase mutant containing only the central catalytic domain was also inhibited, indicating that the binding site for these compounds resides in the central 50−212 amino acids of HIV-1 integrase. Binding at or near the integrase catalytic site was also suggested by a global inhibition of the choice of attacking nucleophile in the 3′-processing reaction. NSC 158393 inhibited HIV-2, feline, and simian immunodeficiency virus integrases while eukaryotic topoisomerase I was inhibited at higher concentrations, suggesting selective inhibition of retroviral integrases. Molecular modeling studies revealed that the two hydroxyls and two carbonyl moieties in NSC 158393 may represent essential elements of the pharmacophore. Antiviral efficacy was observed with NSC 158393 derivatives that inhibited both HIV protease and integrase, and the most potent integrase inhibitors also inhibited HIV protease. Hydroxycoumarins may provide lead compounds for development of novel antiviral agents based upon the concurrent inhibition of two viral targets, HIV-1 integrase and protease.

Introduction

The three viral enzymes encoded by the *pol* gene of human immunodeficiency virus (HIV) play key roles in the virus replication cycle. Two of these enzymes, reverse transcriptase and protease, have been the focus of intense research as targets for chemotherapeutic intervention.[1] Research is now in progress to develop inhibitors of HIV integrase.[2−6]

Retroviruses encode the integrase protein at the 3′-end of the *pol* gene. This enzyme, an HIV protease cleavage product of the *gag−pol* fusion protein precursor, is contained in the virus particle and is required for viral replication.[7,8] It catalyzes the integration of a double-stranded DNA copy of the RNA genome, synthesized by reverse transcriptase, into a host chromosome in a two-step reaction. First, integrase cleaves the last two nucleotides from each 3′-end of the linear viral DNA, leaving the terminal dinucleotide CA-3′-OH.[9,10] This activity is referred to as the 3′-processing or dinucleotide cleavage (Scheme 1) . Secondly, after transport to the nucleus as a nucleoprotein complex, integrase catalyzes a DNA strand transfer reaction involving a nucleophilic attack from the cleaved 3′-ends to a host chromosome. [For recent reviews, see Katz and Skalka[11] and Vink and Plasterk.[12]]

In the present study, we have examined the effects on HIV-1 integrase of compounds recently described as protease inhibitors. All of the compounds tested are novel with respect to previously described integrase inhibitors. For example, they lack key structures found in previous inhibitors such as *o*-hydroxyl groups on the aromatic rings,[2,3] polysulfonate groups,[4] and nucleotide monophosphate groups.[5] In light of the observed inhibition of both integrase and protease, the selectivity for HIV-1 integrase was probed by assaying for the inhibition of related lentiviral integrases and mammalian topoisomerase I. Molecular modeling studies and structure−activity relationships were employed to evaluate the molecular mechanism through which these compounds exert their inhibitory activity.

Results and Discussion

Inhibition of HIV-1 Integrase. The structures of the two lead compounds used in this study are shown in Figure 1, and their inhibition of various activities is summarized in Table 1. NSC 158393 and 117027 were initially investigated in a dual assay which measures the extents of both 3′-processing and strand transfer using a blunt-ended 21mer duplex oligonucleotide. The 3′-processing reaction liberates the 3′ terminal dinucleotide, producing a 19mer oligonucleotide from a 21mer duplex substrate (Scheme 1). The strand transfer reaction is an isoenergetic transesterification resulting in the insertion of one 3′-processed oligonucleotide into another oligonucleotide, yielding higher molecular weight

* To whom correspondence should be addressed. Phone: (301) 496-5944. Fax: (301) 402-0752.
† Laboratory of Medicinal Chemistry, NCI.
‡ NCI−Frederick Cancer Research and Development Center.
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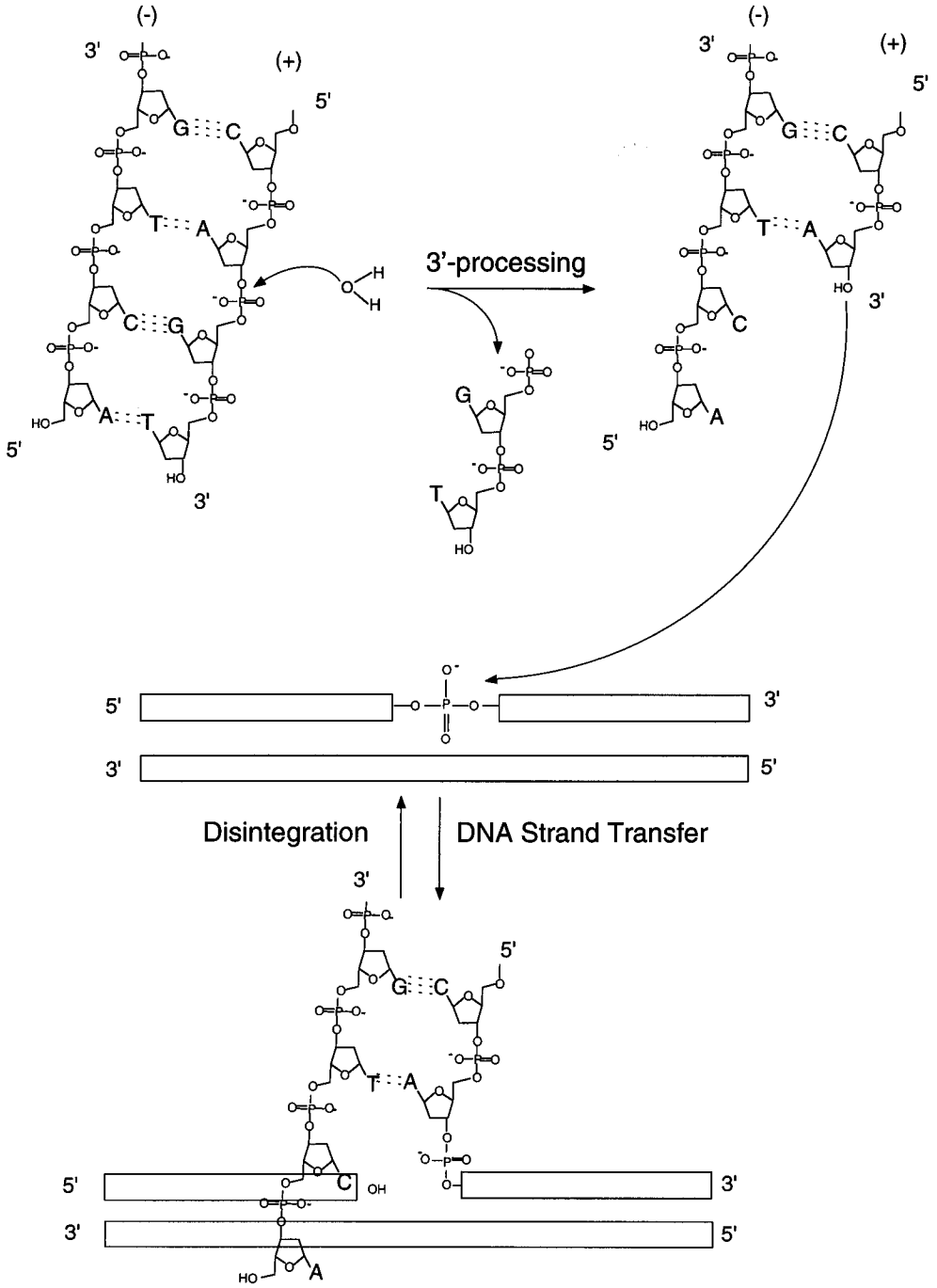
Scheme 1. Reaction Mechanisms for the 3′-Processing, DNA Strand Transfer, and Disintegration Reactions Catalyzed by HIV-1 Integrase[a]



[a] Water is shown as the nucleophile for the 3′-processing reaction. However, *in vitro* it can be either water, glycerol, or the hydroxyl end of the viral DNA.[13,17] In the DNA strand transfer, the attack on the phosphodiester bond shown is only to demonstrate the chemical mechanism and is not meant to imply any sequence specificity for that site.

species with slower migration than the 21mer substrate. As shown in Figure 2A, both compounds showed significant inhibition of both strand transfer and 3′-processing at 2 μM (lanes 5 and 9). NSC 158393 was 3−5-fold more potent than NSC 117027 (Figures 1 and 2A).

In order to investigate whether NSC 158393 inhibited strand transfer directly or as a result of 3′-processing inhibition, this compound was used in an assay which measures only strand transfer. This assay uses a "precleaved" substrate in which a 19mer lacking the terminal GT dinucleotide is annealed to the complementary 21mer. The results (Figure 2B, IC_{50} of 3 μM) demonstrate that NSC 158393 inhibits the DNA strand transfer step of the integration reaction (Figure 2A, lanes 9−11). The finding that this compound has a higher IC_{50} value for strand transfer using the precleaved substrate suggests that it is more active on the first step (3′-processing) of the integration reaction.

Mechanism and Site of Inhibition. (A) Global Inhibition of All Nucleophiles in the 3′-Processing Reaction. As the effect of these compounds was primarily on 3′-processing, this reaction was further investigated using a substrate DNA labeled at the 3′-end. Retroviral integrases can use different nucleo-

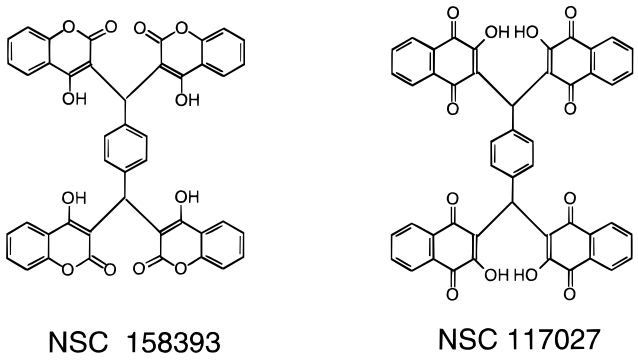


Figure 1. Chemical structures of NSC 158393 and 117027.

Table 1. Inhibition of Various Enzymatic Activities by NSC 158393 and 117027

	IC50 (μM)	
activity	NSC 158393	NSC 117027
HIV-1 IN 3′-proc	1.5 ± 0.5	7.5 ± 2
HIV-1 IN ST	0.8 ± 0.3	2.7 ± 0.9
HIV-2 IN 3′-proc	1	na[a]
FIV IN 3′-proc	2.2	na
SIV IN 3′-proc	2.3	na
topoisomerase I	10	60
HIV-1 protease	1.7	0.75
HIV-1 infected CEM cells	11.5	inactive

[a] na, data not available.

philes in the 3′-processing reaction.[13,17] For convenience of radiolabeling, the substrate DNA was extended by one base pair using [α-^{32}P]cordycepin triphosphate. Recombinant integrases *in vitro* use water, glycerol, or the hydroxyl group of the viral DNA terminus, yielding a linear trinucleotide with a 5′-phosphate (L in Figure 3A), a linear trinucleotide with a glycerol esterified to the 5′-phosphate (G), or a cyclic trinucleotide (C), respectively. NSC 158393 inhibited glycerolysis, hydrolysis, and cyclic nucleotide formation to the same extent (Figure 3A). These data suggest that there is an indiscriminate block of nucleophilic attack in the 3′-processing reaction, suggestive of drug binding at the HIV-1 integrase active site.

(B) Disintegration Reactions with HIV-1 Integrase Deletion Mutants. The HIV-1 integrase catalytic site is located in the central core domain of the enzyme. It consists of three essential acidic residues (two aspartates and one glutamate) which are conserved among retrotransposases and retroviral integrases.[12,18] Integrase can catalyze *in vitro* an apparent reversal of the DNA strand transfer reaction, called disintegration.[15] In contrast to the 3′-processing and strand transfer reactions, disintegration does not require either the N-terminal zinc-finger region or the C-terminal DNA-binding domain of integrase.[14]

In order to determine whether NSC 158393 binds to and inhibits the central catalytic domain, the disintegration reaction catalyzed by an integrase deletion mutant IN$^{50-212}$ was examined (Figure 3B). Significant inhibition was observed at and above 15 μM (lane 3). The concentration 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 inhibitors.[2] The finding that NSC 158393 is active against the IN$^{50-212}$ mutant implies that its binding to the integrase core region is responsible for integrase inhibition.

(C) UV Cross-Linking of 5′-Labeled Substrate DNA. In order to ascertain whether DNA binding was also affected by these compounds, UV cross-linking of integrase−DNA reactions were performed. Cross-linking of substrate DNA to integrase followed by electrophoresis results in a product having a molecular weight of approximately 39 kDa.[19] As seen in Figure 3C, NSC 158393 and 117027 inhibited binding of HIV-1 integrase to its substrate DNA. Significant inhibition of binding was seen in the presence of 1.5 μM NSC 158393 (lane 4) or 5 μM NSC 117027 (lane 8). These concentrations are close to the IC_{50} values for 3′-processing and strand transfer (Table 1), suggesting that inhibition of integrase activity may occur through inhibition of DNA binding.

Selectivity of Inhibition by NSC 158393. NSC 158393 was tested for inhibition of the related retroviral integrases from HIV-2, simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV).[18] As seen in Figure 4 and Table 1, NSC 158393 inhibited 3′-processing catalyzed by all the retroviral integrases

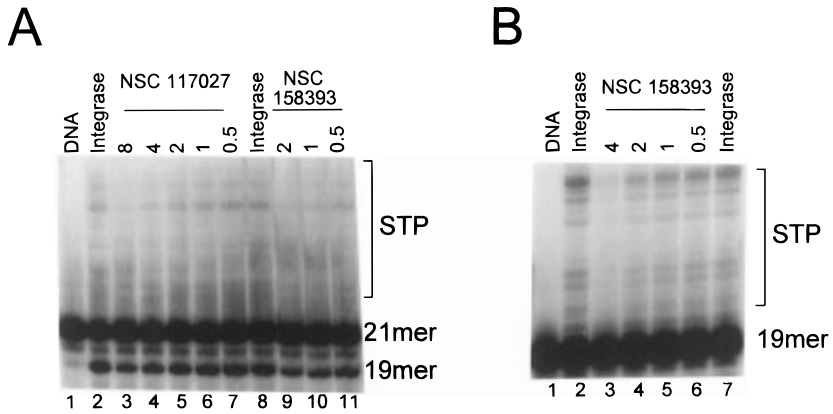


Figure 2. Inhibition of HIV-1 integrase-catalyzed 3′-processing and strand transfer by NSC 158393 and 117027. (A) Phosphorimager picture showing 3′-processing and strand transfer reactions. Lane 1, DNA alone; lanes 2 and 8, plus integrase; lanes 3−7, plus integrase in the presence of NSC 117027; lanes 9−11, plus integrase in the presence of NSC 158393. Drug concentrations in micromolar are indicated above each lane. The DNA strand transfer products (STP) are indicated by the bracket. The 3′-processing product (19mer) and the DNA substrate (21mer) are also shown. (B) Phosphorimager picture showing inhibition of strand transfer using the precleaved oligonucleotide (19mer substrate). Lane 1, DNA alone; lanes 2 and 7, plus integrase; lanes 3−6, plus integrase in the presence of NSC 158393.

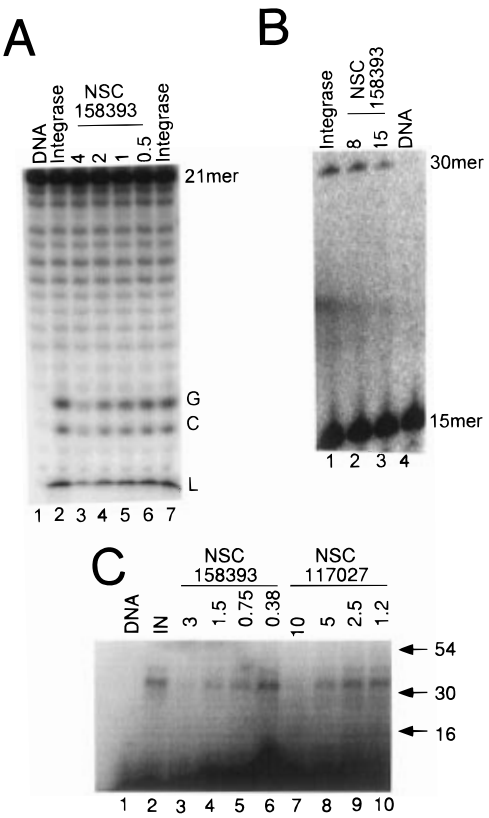


Figure 3. Mechanism and site of inhibition of HIV-1 integrase by NSC 158393 and 117027. (A) Inhibition of HIV-1 integrase-catalyzed 3′-processing using the 3′-end-labeled substrate. Phosphorimager picture showing a typical experiment. Lane 1, DNA alone; lanes 2 and 7, with integrase; lanes 3−6, in the presence of the indicated concentrations of NSC 158393. Recombinant integrases *in vitro* use water, glycerol, or the hydroxyl group of the viral DNA terminus, yielding a linear trinucleotide with a 5′-phosphate (L in Figure 3A), a linear trinucleotide with a glycerol esterified to the 5′-phosphate (G), or a cyclic trinucleotide (C), respectively. (B) Inhibition of disintegration catalyzed by the deletion mutant IN^{50-212}. Phosphorimager picture showing a typical experiment. Lane 1, with IN^{50-212}; lanes 2 and 3, in the presence of the indicated concentrations of NSC 158393; lane 4, DNA alone. (C) Inhibition of the DNA binding activity of HIV-1 integrase. Lane 1, DNA alone; lane 2, with integrase; lanes 3−6, in the presence of the indicated concentrations of NSC 158393; lanes 7−10, in the presence of the indicated concentrations of NSC 117027. The migrations of proteins of known molecular mass (in kDa) are shown to the right of the gel.

tested: HIV-2 (IC_{50} = 1 μM), SIV (IC_{50} = 2.3 μM), and FIV (IC_{50} = 2.2 μM).

In order to determine whether inhibition by these compounds extended beyond the retroviral integrases to other types of DNA binding enzymes, NSC 158393 and 117027 were tested for inhibition of eukaryotic topoisomerase I. An oligonucleotide bearing a unique strong topoisomerase I cleavage site in its center was used.[16] The ability of each of these compounds to either induce cleavable complexes or inhibit the formation of the cleavable complex induced by camptothecin[20] was assayed. Neither compound trapped cleavable complexes (data not shown), but both were able to inhibit the ability of topoisomerase I to generate camptothecin-mediated cleavable complex (Figure 5) at 10−20-fold higher drug concentrations (Table 1) than those observed for the retroviral integrases. These results suggest that NSC 158393 and 117027 can inhibit other DNA binding enzymes, although at significantly higher concentrations than those required for retroviral integrases.

Inhibition of HIV-1 Integrase by Structural Analogues of NSC 158393 and 117027. Both compounds were tested in the *in vitro* anti-HIV screen. The results are presented in Table 1. Analogues of these two lead compounds were obtained by searching the NCI 3D database. All of the analogues that resulted from our search were tested first in the anti-HIV screen and then for inhibition of both HIV-1 protease and HIV-1 integrase. Their structures and activities are shown in Tables 2−4. From Table 2, it is clear that while compounds containing a single 4-hydroxycoumarin ring (e.g., NSC 251150, 251152, and 647086) are capable of inhibiting HIV-1 protease at micromolar concentrations, the presence of two coumarin rings is essential for inhibition of HIV-1 integrase. For example, among the 10 compounds containing a single 4-hydroxycoumarin ring in Table 2, none displayed significant inhibitory activity against HIV-1 integrase even at 100 μM. For the compounds containing two 4-hydroxycoumarin rings, however, moderate activity was observed when a phenyl linker was present. For example, NSC 373937 exhibited a 20-fold decrease in potency compared to NSC 158393. Interestingly, NSC 32180, which also has only two 4-hydroxycoumarin rings but also has additional hydroxy and *n*-hexyl substituents, was only 10-fold less potent than NSC 158393. The same results were observed when analogues containing the 2-hydroxy quinone ring were tested (Tables 3 and 4). For example, NSC 92202, 117272, and 125292, which have only two quinone rings, exhibited 7−10-fold decreased potency compared to NSC 117027. Once again, in this series, additional substituents on the phenyl linker increased potency (compare NSC 117272 or 125292 to NSC 92202). These data suggest that the size (i.e., two to four ring systems) of these derivatives may be important for activity. For example, compounds having two or four rings are potent regardless of whether the ring is a 4-hydroxycoumarin or 2-hydroxy quinone. Conversely, compounds consisting of only one ring exhibit no inhibitory activity. These results are in agreement with those obtained using coumermycin A1 and analogues as inhibitors of HIV-1 integrase.[21] Coumermycin A1, a dimeric compound, was found to be active in the 10 μM range while all of its monomeric analogues were inactive at 100 μM. Compounds are currently being designed, synthesized, and tested on the basis of these data. For example, studies are in progress to discern the relative contributions of hydroxyl and hexyl substituents on the hydroxycoumarin ring and of linker hydrophobicity and size using designed synthetic derivatives and molecular modeling. These data will be presented in the future.

Comparisons to HIV-1 Protease. Analogues of NSC 158393 were also tested against HIV-1 protease, and their antiviral efficacy was determined. The results are presented in Tables 2−4. NSC 158393 was the best integrase inhibitor, was among the best protease inhibitors, and was the only compound which showed antiviral activity. Generally, the inhibitory activity against HIV-1 protease was found to be a good qualitative predictor for the inhibitory activity against HIV-1 integrase. For example, NSC 32180, 117027, and 158393 were both integrase and protease inhibitors. The

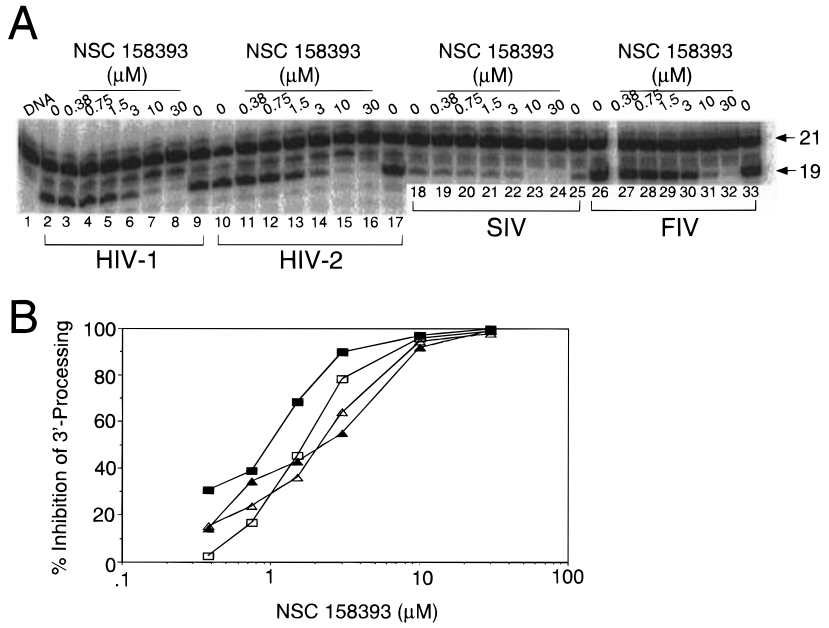


Figure 4. Inhibition of other retroviral integrases by NSC 158393. (A) Phosphorimager picture showing inhibition of HIV-1, HIV-2, FIV, and SIV integrases. Lane 1, DNA alone; lane 0 is for integrase without drug; concentrations in lanes 3−8, 11−16, 19−24, and 27−32 were 0.38, 0.75, 1.5, 3, 10 and 30 μM. The 3′-processing product (19mer, lower arrow) and the DNA substrate (21mer, upper arrow) are shown. (B) Graph showing the inhibition of 3′-processing catalyzed by the various retroviral integrases. Inhibition of HIV-1, HIV-2, SIV, and FIV are depicted by the open squares, filled squares, filled triangles, and open triangles, respectively.

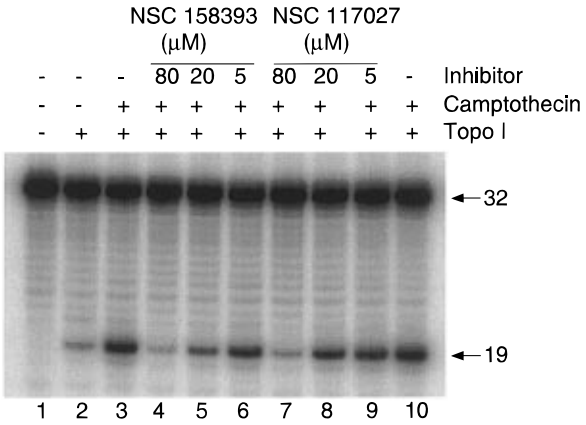


Figure 5. Inhibition of mammalian topoisomerase I (topo I) cleavable complex formation by NSC 158393 and 117027. Lane 1, DNA alone; lane 2, with topo I; lanes 3 and 10, with topo I and camptothecin; lanes 4−6, same plus the indicated concentrations of NSC 158393; lanes 7-9, same but with the indicated concentrations of NSC 117027.

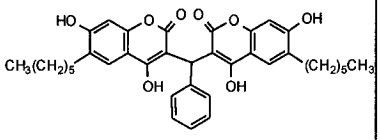
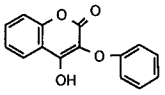
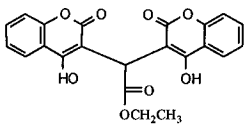
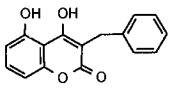
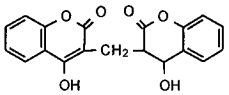
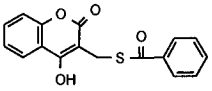
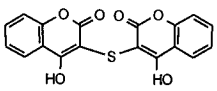
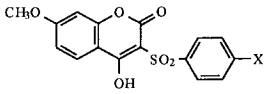
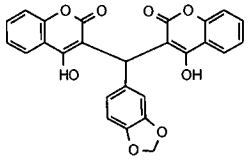
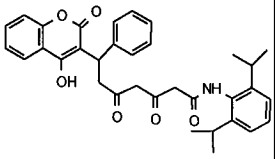
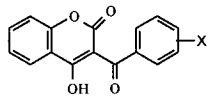
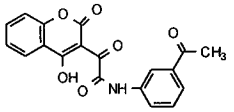
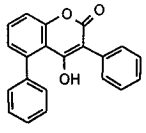
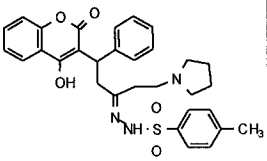
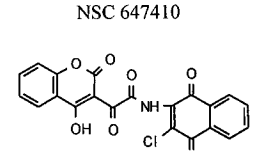
same correlation was observed with compounds which were moderate integrase and protease inhibitors. However, there was no linear relationship between the IC_{50} values for protease and integrase inhibition for these 4-hydroxycoumarin analogues. For example, NSC 32180 ($IC_{50} = 0.32$ μM) was found to be 5-fold more potent than NSC 158393 ($IC_{50} = 1.7$ μM) in the HIV-1 protease assay but 10-fold less potent than NSC 158393 in both the HIV-1 integrase 3′-processing and strand transfer assays (Table 1).

In the 2-hydroxy quinone series (Tables 3 and 4), very similar structure−activity relationships were observed. Among the 2-hydroxy quinones containing a single 2-hydroxy quinone ring in Table 4, although four out of six compounds (NSC 2035, 108700, 129083, 129153) exhibited significant activity against HIV-1 protease, only one compound, NSC 129083, showed weak activity against HIV-1 integrase, with IC_{50} values of 92 and 56 μM in 3′-processing and strand transfer assays, respectively. However, for compounds containing two 2-hydroxy quinone rings, 8 out of 13 compounds showed significant inhibitory activity against HIV-1 integrase. It is noted that additional hydrophobic moieties in these analogues may enhance their activity against HIV-1 integrase (compare NSC 92073 with 92202 and NSC 117271 with 117272).

Molecular Modeling and Mechanism of Inhibition. Taken together, our results suggest that among compounds containing either 4-hydroxycoumarin or 2-hydroxy quinone, two hydroxycoumarin or two hydroxy quinone rings are essential for activity against HIV-1 integrase. Therefore, the two rings in these compounds should contain the crucial "pharmacophore" elements. Furthermore, since these two classes of HIV-1 integrase inhibitors showed strikingly similar structure−activity relationships, it is likely that their 3D pharmacophores (the arrangement of atoms essential to enzyme binding) may be well overlapped in space. Molecular modeling studies were thus employed to investigate these hypotheses.

Five thousand conformations of both NSC 158393 and 117027 were randomly generated and subsequently minimized and compared using molecular modeling package QUANTA/CHARMM. Among the low-energy conformations, NSC 158393 and NSC 117027 were found to have excellent overlaps with the two hydroxyl and two carbonyl groups, in terms of both their locations and orientations (Figure 6). The overall root-mean-square value of rigid-body fitting of the four oxygens in NSC 158393 to those in 117027 is 0.53 Å. This excellent fit suggests that these four oxygens, or at least some of them, may be the essential components of a pharmacophore common to both compounds and should play important roles in the specific hydrophilic/hydrogen bond interactions with HIV-1 integrase enzyme. Other

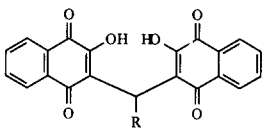
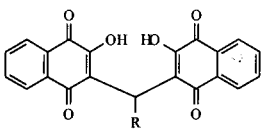
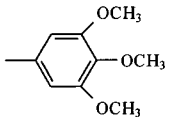
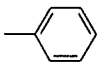
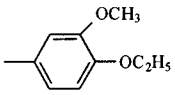
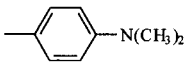
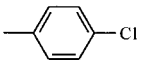
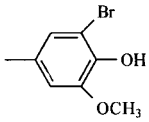
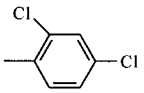
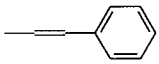
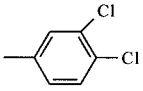
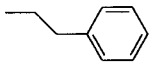
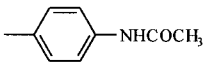
Table 2. Inhibition of HIV-1 Integrase and Protease by a Series of Compounds Containing at Least One 4-Hydroxycoumarin Ring

	IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR		IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR
 NSC 32180	10.5	12.0	0.32	 NSC 251152	>100	>100	32
 NSC 36366	>100	>100	>100	 NSC 251156	>100	>100	>100
 NSC 41834	>100	>100	>100	 NSC 280795	>100	>100	>100
 NSC 41234	80	100	21	 NSC 619387 (X=Cl) NSC 619389 (X=CH_3)	>100	>100	>100
 NSC 373937	47	18	30	 NSC 647086	>100	>100	31
 NSC 133800 (X=3-NO_2) NSC 133806 (X=4-Cl)	>100	>100	>100	 NSC 648626	>100	>100	>100
 NSC 251150	>100	>100	13	 NSC 649811	>100	>100	>100
				 NSC 647410	>100	>100	>100

portions of the molecules may be important for nonspecific hydrophobic interactions. The HIV-1 integrase "pharmacophore" proposed here could well explain the structure−activity relationships for these new HIV-1 integrase inhibitors (Tables 2−4) and provide important clues to the design of new, more potent HIV-1 integase inhibitors.

How could these oxygens be involved in inhibition of the DNA-binding and catalytic activities of HIV-1 integrase? Site-directed mutagenesis and sequence alignment have identified three amino acid residues in the catalytic core which are conserved among all retroviral integrases[18] and are critical for activity.[22] These are Asp64, Asp116, and Glu152. Such acidic residues are

Table 3. Inhibition of HIV-1 Integrase and Protease by a Series of Compounds Containing at Least One 2-Hydroxyquinone Ring[a]

 R =	IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR	 R =	IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR
—CH_3 NSC 92073	>100	>100	n.a.	 NSC 117278	>100	>100	n.a.
 NSC 92202	68	48	n.a.	 NSC 117279	>100	>100	n.a.
 NSC 92203	>100	>100	n.a.	—$COOC_2H_5$ NSC 120048	92	60	n.a.
 NSC 117271	>100	>100	n.a.	 NSC 125292	32	20	n.a.
 NSC 117272	37	40	24	 NSC 125298	>100	72	n.a.
 NSC 117275	90	52	n.a.	 NSC 125296	83	90	n.a.
 NSC 117276	86	78	n.a.				

[a] na, data not available.

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.[23] Therefore, these oxygens on the 4-hydroxycoumarin or 2-hydroxy quinone rings could become coordinated to the essential divalent metal ion, disrupting its coordination to the enzyme or enzyme—DNA complex. The compounds could also be involved in hydrogen bonding with the essential acidic residues at the active site, interfering with their role(s) in catalysis. For example, coordination of the ring oxygens to either the metal ion or the carboxylate groups present in the integrase active site could inhibit binding of the DNA phosphodiester backbone such that an appropriately positioned transition state could not be generated. Such a hypothesis has been proposed for the inhibition of HIV-1 integrase by the *o*-hydroxyl groups present on aromatic rings in flavones[24] and tyrphostins.[2]

Dual Inhibition of Viral Enzymes as a New Stratagem in AIDS Drug Development. Our study demonstrates that NSC 158393, which exhibits antiviral activity, inhibits both integrase and protease and that this dual inhibition is observed for other derivatives. Such dual inhibition has also been demonstrated by several other families of compounds. For example, flavones,[24,25] cosalane analogues,[6] and bathocuproine disulfonate[26,27] have been found to inhibit both HIV-1 protease and integrase.

High-dose monotherapy exerts a high selective pressure for the generation of mutant virus. Therefore, clinical benefits are only short term before resistance and decreased drug susceptibility arise. A second option is to treat with a combination of drugs, each at an effective lower dose (decreasing the toxicity associated with either drug), to avoid applying a high selection for resistance. Furthermore, the combined antiviral effect could equal or exceed the results obtained from high-dose monotherapy depending on whether the drugs were additive or synergistic. Moreover, certain agents in the regimen may be more effective in particular tissues and cell types. For example, reverse transcriptase inhibitors, which act at early stages of viral replication, can block *de novo* infection of lymphocytes. However, they are inactive against virus harbored in chronically

Table 4. Inhibition of HIV-1 Integrase and Protease by a Series of Compounds Containing at Least One 2-Hydroxyquinone Ring[a]

R =	IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR	R =	IC50 (μM) 3'-Proc.	IC50 (μM) ST	IC50 (μM) PR
NSC 2035	>100	>100	8.5	NSC 108700	>100	>100	50
—H NSC 8625	>100	>100	>100	NSC 129083	92	56	3
—S— NSC 108029	>100	>100	>100	—NH-$(CH_2)_6CH_3$ NSC 129153	>100	>100	33

[a] na, data not available.

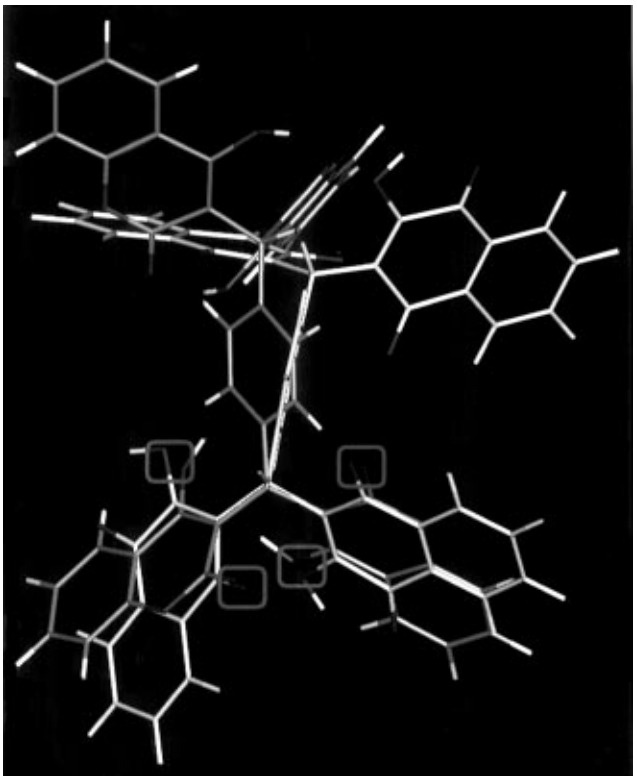


Figure 6. Molecular modeling and superimposition of the energy minimized conformations of NSC 158393 (green) and 117027 (yellow) showing the overlap of four oxygens of the molecules. The overall root-mean-square value of rigid-body fitting of the four oxygens in NSC 158393 to those in 117027 is 0.53 Å.

infected cells such as monocytes and macrophages (which have a long lifetime, produce a large amount of virus per cell, and efficiently spread HIV infection to lymphocytes via cell-to-cell virus transfer). This lack of activity, which arises from reverse transcription no longer being required for viral replication once the provirus is already integrated into the host chromosome, along with the emergence of drug resistance, may account in part for their limited activity in patients treated for long durations. Therefore, antiretrovirals capable of blocking the virus at late stages of its replication cycle, and perhaps in chronically infected cells, are important in HIV therapy.[28]

Experimental Section

Compounds. All of the compounds in this study were obtained from the NCI Repository (Drug Synthesis and Chemistry Branch, NCI).

Preparation of Radiolabeled DNA Substrates. The following HPLC purified oligonucleotides were purchased from Midland Certified Reagent Company (Midland, TX): AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE157, 5′-GAAAGCGACCGCGCC-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGC-GTCC-3′; AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′; RM22M, 5′-TACTGCTAGAGATTTTCCACAC-3′. AE117, AE118, and the first 19 nucleotides of AE156, correspond to the U5 end of the HIV-1 long-terminal repeat (LTR).

To analyze the extents of 3′-processing and strand transfer using 5′-end-labeled substrates,[9,10] 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 extents of 3′-processing and strand transfer using 3′-end-labeled substrates, AE118 was 3′-end-labeled using α-[^{32}P]cordycepin triphosphate (Dupont-NEN) and terminal transferase (Boehringer Mannheim). The transferase was heat-inactivated, and RM22M 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 spin column as before.

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

Integrase Proteins. Purified recombinant wild-type HIV-1 integrase,[13] deletion mutant HIV-1 IN$^{50-212}$,[14] and purified recombinant wild-type simian immunodeficiency virus integrase were generous gifts of Drs. R. Craigie and A. Hickman, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. The plasmid encoding the HIV-2 integrase was kindly provided by Dr. Ronald H. A. Plasterk, Netherlands Cancer Institute, The Netherlands. Purified recombinant wild-type feline immunodeficiency virus integrase was kindly provided by Dr. Samson Chow, UCLA School of Medicine, Los Angeles, CA.

3′-Processing, Strand Transfer, and Disintegration Assays. Integrase was preincubated at a final concentration of 200 (for HIV-1 and HIV-2) or 600 (for SIV and FIV) nM with inhibitor 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) at 30 °C for 30 min. The 5′-end ^{32}P-labeled oligonucleotide substrate (20 nM) was then added, and incubation was continued for an additional 60 min. The final reaction volume was 16 μL.

Disintegration reactions[15] were performed using the Y oligonucleotide (i.e., the branched substrate in which the U5 end was "integrated" into target DNA) instead of the linear viral U5 oligonucleotide.

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, 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, 8M urea). After drying, gels were exposed and analyzed using a Molecular Dynamics phosphorimager (Sunnyvale, CA).

UV Cross-Linking Experiments. Integrase was preincubated with inhibitor for 30 min and then incubated with substrate in reaction buffer as above for 5 min at 30 °C. Reactions were then irradiated with a UV transilluminator (254 nm wavelength) from 3 cm above (2.4 mW/cm^2) at room temperature for 10 min. An equal volume (16 μL) of 2X SDS-PAGE buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) was added to each reaction, and the reaction 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.

Topoisomerase Reactions. Reactions were performed in 10 μL of reaction buffer (0.01 M Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml bovine serum albumin) with the following duplex oligonucleotide substrate labeled with α-[^{32}P]cordycepin at the 3′-end of the upper strand (asterisk).[16]

5′-GATCTAAAAGACTT∧GGAAAAATTTTTAAAAAA*

ATTTTCTGAA-CCTTTTTAAAAATTTTTTCTAG-5′

This oligonucleotide contains a single topoisomerase I cleavage (caret on the upper strand). Approximately 50 fmol oligonucleotide per reaction were incubated with 10 units of calf thymus DNA topisomerase I (Gibco BRL, Gaithersburg, MD). Reactions were stopped by adding sodium dodecyl sulfate (0.5% as final concentrations). Proteinase K (ICN Biochemicals, Cleveland, OH) (0.5 mg/mL final concentration) was then added to the reaction mixtures, and proteolysis was carried out for an additional 60 min at 50 °C. Proteolysis was halted by the addition of 36 μL of 2.5 X loading buffer (98% formamide, 0.01 M EDTA, 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue). Electrophoresis on a denaturing 16% polyacrylamide gel and quantitation were performed as described above.

In Vitro Anti-HIV Assay. Anti-HIV screening of test compounds was performed using the XTT cytoprotection assay as previously described.[29] This cell-based microtiter assay quantitates the drug-induced protection from the cytopathic effect of HIV-1_{RF} on CEM (CD4+ T-cell line) cells.

Acknowledgment. We would like to thank Drs. R. Craigie and A. Hickman (Laboratory of Molecular Biology, NIDDK) for generously providing us with purified recombinant wild-type HIV-1 integrase, deletion mutant HIV-1 IN$^{50-212}$, purified recombinant wild-type simian immunodeficiency virus integrase, the plasmid encoding the HIV-1 integrase, and advice regarding protein purification. We would also like to thank Drs. R. H. A. Plasterk (Netherlands Cancer Institute) and S. Chow (UCLA) for providing the plasmid encoding the HIV-2 integrase and purified recombinant wild-type feline immunodeficiency virus integrase, respectively. We profitted from stimulating discussions during the course of these experiments with Dr. Kurt W. Kohn (chief, Laboratory of Molecular Pharmacology, NCI). This project was supported by a grant from the NIH Intramural AIDS Targeted Antiviral Program.

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