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Identification of HIV-1 Integrase Inhibitors via Three-Dimensional Database Searching Using ASV and HIV-1 Integrases as Targets

I-Jen Chen,[a] Nouri Neamati,[b,*,†] Marc C. Nicklaus,[b] Ann Orr,[b] Lynne Anderson,[b] Joseph J. Barchi Jr.,[b] James A. Kelley,[b] Yves Pommier[b] and Alexander D. MacKerell Jr.[a,*]

[a]*Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, 20 N. Pine Street, Baltimore, MD 21201, USA*

[b]*Laboratories of Molecular Pharmacology and Medicinal Chemistry, National Cancer Institute, Bethesda, MD 20892, USA*

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Abstract—Integration of viral DNA into the host cell genome is a critical step in the life cycle of HIV. This essential reaction is catalyzed by integrase (IN) through two steps, 3′-processing and DNA strand transfer. Integrase is an attractive target for drug design because there is no known cellular analogue and integration is essential for successful replication of HIV. A computational three-dimensional (3-D) database search was used to identify novel HIV-1 integrase inhibitors. Starting from the previously identified Y3 (4-acetylamino-5-hydroxynaphthalene-2,7-disulfonic acid) binding site on the avian sarcoma virus integrase (ASV IN), a preliminary search of all compounds in the nonproprietary, open part of the National Cancer Institute 3-D database yielded a collection of 3100 compounds. A more rigorous scoring method was used to rescreen the 3100 compounds against both ASV IN and HIV-1 IN. Twenty-two of those compounds were selected for inhibition assays against HIV-1 IN. Thirteen of the 22 showed inhibitory activity against HIV-1 IN at concentrations less than 200 μM and three of them showed antiviral activities in HIV-1 infected CEM cells with effective concentrations (EC_{50}) ranging from 0.8 to 200 μM. Analysis of the computer-generated binding modes of the active compounds to HIV-1 IN showed that simultaneous interaction with the Y3 site and the catalytic site is possible. In addition, interactions between the active compounds and the flexible loop involved in the binding of DNA by IN are indicated to occur. The structural details and the unique binding motif between the HIV-1 IN and its inhibitors identified in the present work may contribute to the future development of IN inhibitors.

Introduction

Current treatments of acquired immunodeficiency syndrome (AIDS) focus on disruption of reverse transcription of the single-stranded viral RNA or proteolytic cleavage and release of mature viral proteins. The efficacy of these drugs is, unfortunately, limited by quickly developed resistance[1] via mutation of the enzymes which catalyze these functions, reverse transcriptase[2,3] and protease,[4,5] respectively. The current strategy to delay the onset of resistance is combination therapy. This approach uses a combination of antiretroviral drugs, thereby decreasing the potential of single point mutations in HIV overcoming the therapeutic regimen. Combination therapy has been shown to reduce AIDS-related morbidity and mortality dramatically.[6,7] Viral eradication, however, is still not achieved,[8] requiring long-term use of combination drug therapy. To further enhance the effectiveness of combination therapy, drugs targeting other steps in the life cycle of HIV are needed.

An essential step in the viral life cycle is integration of the viral DNA into the host genome.[9] This step is catalyzed by the viral enzyme, HIV integrase (HIV IN). Integrates catalyzes two separate but chemically similar reactions, known as 3′-processing and DNA strand transfer.[10,11] In 3′-processing, IN removes a dinucleotide next to a conserved cytosine-adenine sequence from each 3′-end of the viral DNA.[12,13] IN then attaches the processed 3′-ends of the viral DNA to the host cell DNA[12,14] in the strand transfer reaction. The essential

Abbreviations: 3-D, three-dimensional; HIV-1, human immunodeficiency virus type 1; IN, integrase; RMS, root-mean-square; Y3, 4-acetylamino-5-hydroxynaphthalene-2,7-disulfonic acid; Y4, 4-acetylamino-5-hydroxynaphthalene-2-sulfonic acid; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

*Corresponding authors. D.M. Tel.: +1-410-706-7442; fax: +1-410-706-0346; e-mail: amackere@rx.umaryland.edu N.N. Tel.: +1-301-435-2463; fax: +1-301-402-0752; e-mail: neamati@nih.gov

†New address: University of Southern California, School of Pharmacy, 1985 Zonal Avenue, Room PSC 304A, Los Angeles, CA 90089-9121, USA. Tel.: +1-323-442-2341; fax: +1-323-442-1390; e-mail: neamati@usc.edu

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role of IN has been shown in experiments where IN mutants with defective catalytic capability are replication incompetent.[9,15,16] As there is no known human counterpart of HIV integrase, IN is a good target for drug design.

Because of the potential of IN inhibitors as therapeutic agents, significant efforts have been devoted to the identification of IN inhibitor lead compounds.[17,18] Searching for IN inhibitors has involved testing of compounds that inhibit other enzymes with similar substrates or possessing similar mechanisms to IN,[19,20] conducting structure–activity relationship studies of known active compounds,[21–23] and performing database searches to match pharmacophores identified based on existing inhibitors[24–26] or just randomly screening.[27] Recently, the first crystal structure of HIV-1 IN complexed with an inhibitor at the catalytic site was solved.[28] To date, the identified inhibitors have not been found to be effective therapeutic agents, in part because many of those compounds are cytotoxic. One of these inhibitors, Y3, a disulfonic acid, was initially discovered via pharmacophore searching[24] of compounds in the National Cancer Institute (NCI) 3-D database.[29] The mechanism of inhibition by Y3 was suggested to possibly involve chelation of the metal ion at the IN catalytic site, referred to as the DD-35-E motif. Y3 was co-crystallized with the avian sarcoma virus integrase (ASV IN)[30] yielding the first IN-inhibitor 3-D structure. Y3 in the complex structure is located in close proximity to the catalytic site of ASV IN; however, it does not directly interact with the catalytic-site residues. At present, there is no structural information of any HIV-1 IN-inhibitor complex that binds in a manner similar to that of Y3 to ASV IN. There is, however, 24% sequence homology[31] between ASV IN and HIV-1 IN and 3-D structural alignment based on the C^{α} atoms reveals a RMS deviation of 1.4 Å between the enzymes.[31,32] Further, residues Ile-60, Gln-62 and Lys-119 that interact directly with Y3 in the Y3-ASV IN complex correspond to residues Ile-60, Gln-62 and His-114 on HIV-1 IN, suggesting that the Y3 binding site is conserved in both integrases. The significance of the Y3 binding site has also been demonstrated by Heuer et al.[33] and Esposito et al.[34] showing that one of the key binding residues, Gln-62, is critical to viral DNA binding and in the proximity of the catalytic site. Thus, the Y3 binding site represents a potential target site on ASV IN and, by extension, HIV-1 IN for the rational design of novel IN inhibitors.

Three-dimensional structural database searching can greatly accelerate the lead generation step within the drug design process.[35–37] Database searching enables structures to be selectively extracted from databases of small molecule 3-D structures based on some type of molecular similarity, complementarity or topology. Among the numerous programs available to perform these types of studies, DOCK[38] has been shown to hold promise as a tool for the identification of potential drugs via database searching.[39]

A database search protocol by DOCK consists of four stages: target preparation, site characterization,[38,40] grid calculation[41,42] and docking.[43] The target structure can be obtained from X-ray crystallography, NMR, or homology modeling. The binding site of interest on the target is filled with a set of spheres generated by DOCK that are complementary to the site. Electrostatic and van der Waals potential fields generated over the binding site are precalculated and stored in a grid. Each ligand is then flexibly docked into the site of interest,[44] oriented to maximize the overlap with the sphere centers, followed by a grid-based energy scoring step to rank the ligands. The successful application of DOCK in structure-based drug design includes the identification of inhibitors for HIV-1 protease,[45,46] thymidylate synthase,[47] influenza hemagglutinin,[48] and parasitic proteases.[49] In the present study, identification of novel HIV-1 IN inhibitors was performed by 3-D site-directed searches against the Y3-binding site of ASV IN and the corresponding site on HIV-1 IN.

Results and Discussion

Summary of docking runs

Potential inhibitors of HIV-1 IN have been identified via computer-based screening of the NCI 3-D database against the Y3 binding site on ASV IN and the putative Y3 binding site on HIV-1 IN. All compounds were initially screened against ASV IN using the computationally efficient scoring approach referred to as method I. From this initial search, 3100 compounds having favorable interaction energies with the ASV IN Y3 binding site were collected. Energy scores of those compounds ranged from −24.1 to −10.6 kcal/mol (Fig. 1(A)). The more rigorous scoring approach, method II, was used to rescreen the 3100 compounds against the Y3 binding site on both ASV IN and HIV-1 IN. For ASV IN, scores of the 3100 compounds ranged from −25.0 to 0.0 kcal/mol (Fig. 1(B)), and for HIV-1 IN score values were between −29.1 kcal/mol and 0.0 (Fig. 1(C)). Interaction energies were generally more favorable with method I versus method II. This appears to be due to the use of the more refined sphere sets in method II, which focus the docking to a region more confined to the Y3 binding site, thereby limiting nonspecific favorable interactions with IN. The ranking of compounds based on method II was used to select compounds for bioassay.

In vitro evaluation of inhibition of HIV-1 IN

After reviewing the top 200 compounds from the ASV IN, HIV-1 IN and overlap lists, 67 compounds were initially selected for IN inhibitory assay. From these 67 compounds, 40 were evaluated for purity using NMR and mass spectroscopy, from which 22 compounds (Fig. 2) were selected for assay and the final analysis. The IN inhibitory activity of the selected 22 compounds is shown in Table 1. The activity of potential inhibitors on both 3′-processing and DNA strand transfer reactions (Fig. 3(A)) was measured using purified recombinant HIV-1 IN, a 21-mer duplex oligonucleotide, and divalent metal ion (Mn^{+2} or Mg^{+2}). All IC_{50} values were

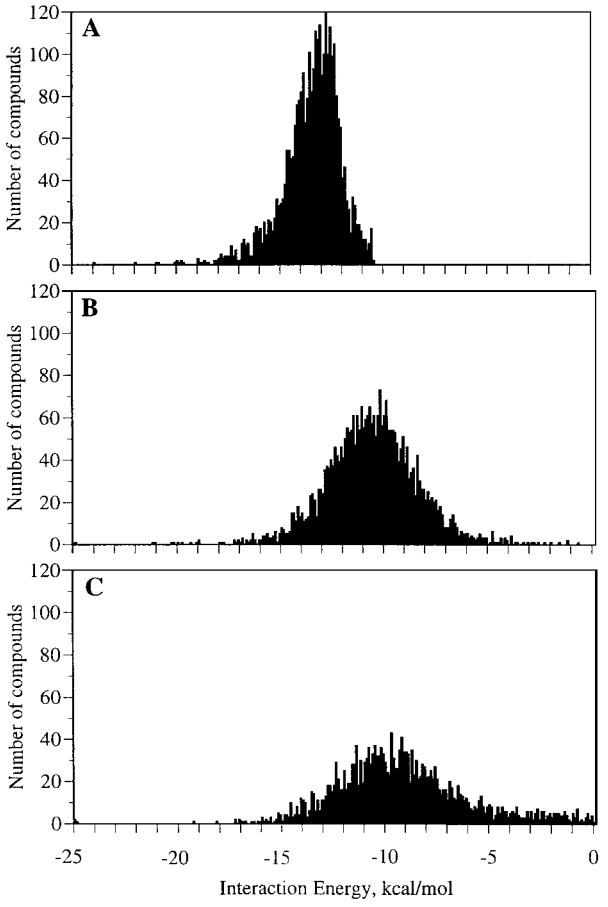


Figure 1. Histograms of IN-inhibitor interaction energy distributions of (A) ASV IN using method I, (B) ASV IN using method II, and (C) HIV-1 IN using method II. Note that energy scores of compounds with positive interaction energies are set to be zero by DOCK.

obtained by measuring inhibitory activity at different concentrations (Fig. 3(B) and (C)).

Of the 22 assayed compounds, 13 showed IN inhibitory activity ($IC_{50} \leq 200$ μM) in the presence of Mn^{+2}, while only eight compounds showed similar activity in the Mg^{+2} containing solution. All compounds which were inactive in the Mn^{+2} containing assays were also inactive in the Mg^{+2} based assays. All the remaining active compounds were more potent in the Mn^{+2} based assays than in the Mg^{+2} based assays by 2- to 4-fold. Compounds **3–5, 8–10, 13,** and **19** were active with both metals. This indicates that chelation of divalent metals in the active site of IN may be responsible for the differential potency of some of these compounds. The metal-dependent activity of those inhibitors in relation to their proposed binding orientations will be discussed below.

Inhibition of HIV-1 IN in cell-based assays

Compounds that were active in either metal based IN inhibition assays were submitted for cell-based antiviral assay. Four of the compounds (**1, 20–22**) that did not exhibit any activity in either metal containing solution were also selected. The antiviral activity of the tested compounds is presented in Table 1 with the dose–response curves for compounds **3** and **8** presented in Figure 4. Three compounds showed cytoprotection, while most compounds in the cell-based assays showed significant toxicity. Three of the compounds (**3**, **8** and **13**) protected the HIV-1 infected CEM cells. Compound **3** was the most potent antiviral compound with an effective concentration (EC_{50}) of 0.8±0.2 μM. However, this compound also showed considerable cytotoxicity (cytotoxic concentration $CC_{50} = 4.4 \pm 0.9$ μM). Compound **8** (Fig. 4) and **13** exhibited similar antiviral and cytotoxicity profiles (Table 1). Both compounds inhibited viral replication at doses that the drugs started to be toxic. Other compounds showed limited toxicity and no antiviral activity could be detected. For example, compounds **4, 7, 12** and **19** with CC_{50} values of 1 to 5 μM against CEM cells were among the most cytotoxic compounds.

Docking method I versus method II

To assess the validity of the more computationally expensive method II versus method I, the ability to correctly dock Y3 (Fig. 2) back in the experimental binding orientation was tested using the two methods. The binding mode of Y3 predicted by method I had an interaction energy with ASV IN of −13.6 kcal/mol. Using method II a more favorable interaction energy of −26.6 kcal/mol was obtained. The RMS deviation of the docked Y3 binding orientation as compared to the experimental structure as predicted by method I was 1.6 Å versus a RMS deviation of 1.1 Å for method II. The RMS deviation of 1.1 Å is comparable with experimental RMS fluctuations of 1.1 Å determined from the crystallographic B-factors indicating that the predicted Y3 orientation from method II is within experimental uncertainty. ASV IN to Y3 contacts were also used to evaluate the quality of the methods. The most important contacts in the Y3-ASV IN crystal structure, the Gln-62 NE2 to sulfonate oxygen and Lys-119 NZ to carbonyl oxygen,[30] were replaced by Gln-62 NE2 to carbonyl oxygen and Lys-119 NZ to sulfonate oxygen for method I, while Gln-62 and Lys-119 both interact with oxygens of one sulfonate for method II. In addition, Y3 in method I lost all hydrophobic interactions with the enzyme, while for method II favorable hydrophobic contacts of the Y3 methyl group to the enzyme (Pro-57 and Leu-58) were present as compared to the X-ray hydrophobic contact of the Y3 methyl group to Ile-60.

Y4, an analogue of Y3 with only one sulfonate group (Fig. 2), has been synthesized elsewhere and tested for IN inhibition activity.[30] While the disulfonate Y3 inhibited 3′-processing of HIV-1 IN at 16.2 μM and strand transfer at 10.9 μM, the single sulfonate Y4 had no activity toward HIV-1 IN inhibition.[30] Docking Y4 into the Y3 site using method II was performed to see if the different IC_{50} values could be explained as well as to further test method II. The Y4 binding mode predicted by method II had an energy of 21.0 kcal/mol, significantly less favorable than the calculated value for Y3. Comparison of the Y3 and Y4 binding orientations to ASV IN from method II showed that the unfavorable interaction energy of Y4 may be due to the missing

sulfonate group, as two oxygens on the additional sulfonate in Y3 also interact with the enzyme (Leu-58 N and Thr-83 O). Also, the hydrophobic naphthalene moiety in Y4 was much closer to the polar residues (Gln-62 and Lys-119) which may be considered repulsive interactions. In addition, the heteroatoms on the amide group of Y4 had less contacts with the enzyme as compared to Y3. Based on the smaller RMS difference of Y3 with respect to the crystal orientation and the ability to predict decreased interactions between Y4 and IN, method II was selected for the final docking run.

Description of the binding regions on ASV IN and HIV-1 IN

In the Y3-ASV IN complex crystal structure,[30] the Y3 binding site is about 7 to 12 Å away from the DD-35-E catalytic site, based on C^{α} to C^{α} distances (Table 2), and located adjacent to the protein crystallographic dimer interface. Two important Y3 binding residues are Gln-62 and Lys-119. Other residues near the Y3 site are mostly nonpolar, including Ile-60, although some additional polar residues are present (e.g., Gln-59 and Trp-61). The proposed Y3 site on HIV-1 IN[32] contains Gln-62 and His-114. They are also ca. 7 to 12 Å (Table 2) from the catalytic site, but not close to the crystallographic dimer interface. Most residues surrounding this site are nonpolar. Residues indicated to contribute to Y3 binding along with the three catalytic residues (Table 2) will be used to discuss different binding modes of selected compounds with both enzymes.

Relevant to the present work is the flexible loop in both ASV IN and HIV IN. This loop is comprised of residues 140 to 154 in ASV IN and has been suggested to be involved in binding of DNA.[33,34,50] In the ASV IN-Y3 complex structure the loop is traced out in the crystal structure, with the exception of residues 147 to 152 which were modeled into the structure.[30] The loop is located between the Y3 and catalytic sites as shown in

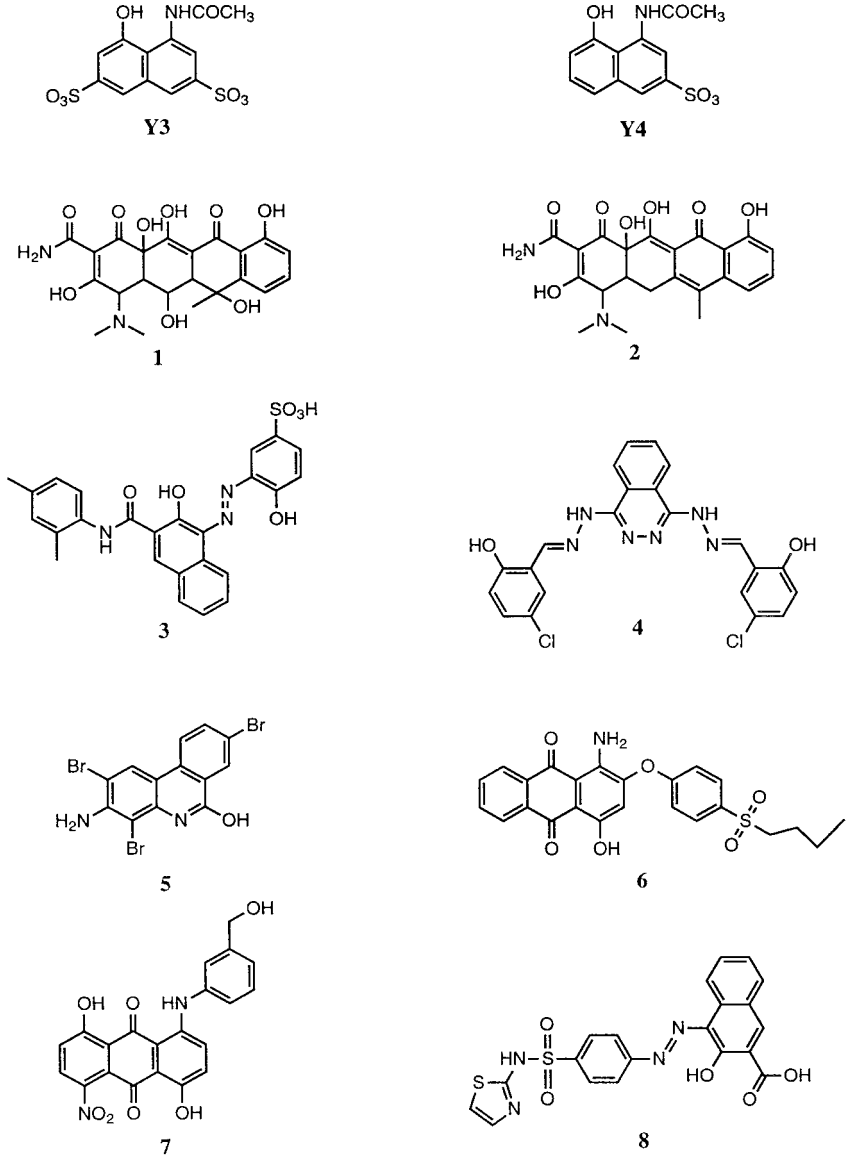
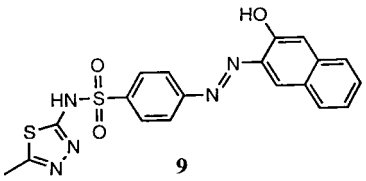


Figure 2(a). Structures of Y3 and Y4 (see abbreviations at start of paper) and compounds subject to IN inhibitory assays. See Table 1 for the corresponding NSC numbers (*continued on next page*).



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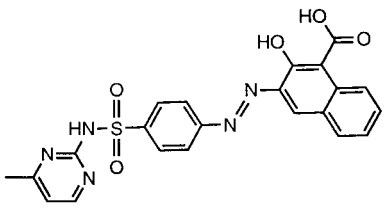

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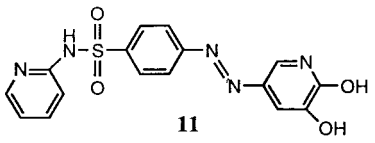

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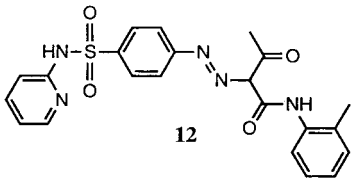

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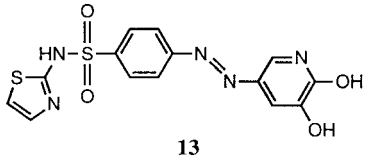

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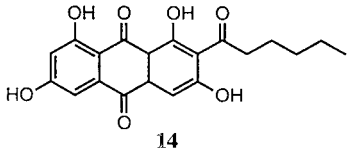

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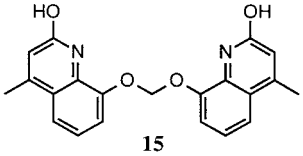

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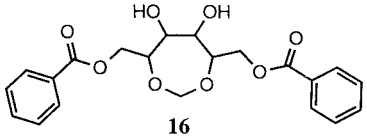

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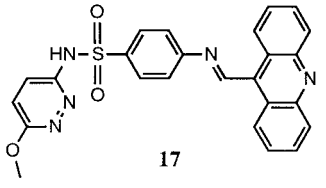

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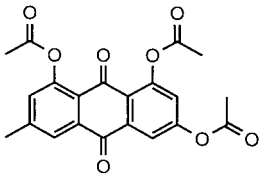

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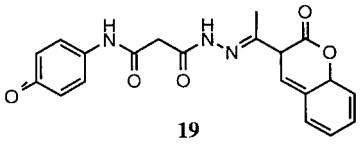

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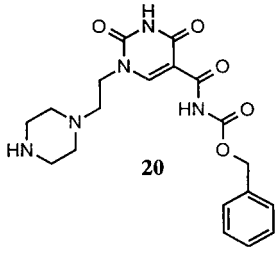

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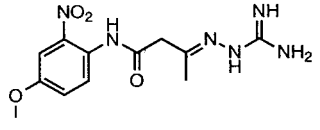

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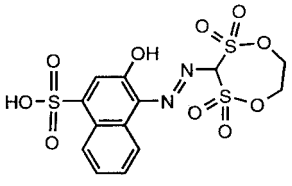

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Figure 2. (*continued*)

Table 1. Results of HIV-1 integrase inhibition and antiviral activity[a]

Compound	NSC No.	IC_{50} (μM)[b] Mn^{+2}		IC_{50} (μM) Mg^{+2}		Antiviral activity	
		3′-Proc	Integration	3′-Proc	Integration	CC_{50} (μM)[c]	EC_{50} (μM)[d]
1	9169	> 200	> 200	> 200	> 200	64	NR[e]
2	44181	23 ± 14	13 ± 3	> 200	> 200	35	NR
3	97318	13 ± 3	14 ± 6	53	22	4.4 ± 0.9	0.8 ± 0.2
4	103668	88 ± 26	105 ± 50	159; 171	143; 150	1.0	NR
5	114690	60 ± 22	69 ± 38	187; 220	156; 200	39	NR
6	127625	> 200	> 200	> 200	> 200	NT[f]	NT
7	128439	32 ± 15	39 ± 22	> 200	> 200	0.7	NR
8	134135	24; 19	46; 40	16; 19	42; 18	162	200[g]
9	134142	39 ± 14	50 ± 8	212	174	23	NR
10	134144	21; 18	44; 20	59; 86	37; 24	10	NR
11	134173	60 ± 6	59 ± 5	269	269	86	NR
12	134191	55; 78	65; 80	> 200	> 200	4.5	NR
13	134215	23 ± 6	21 ± 7	57 ± 19	43 ± 17	196	192
14	138557	30; 53	30; 60	> 200	> 200	65	NR
15	156987	> 200	> 200	> 200	> 200	NT	NT
16	224283	> 200	> 200	> 200	> 200	NT	NT
17	348893	> 200	> 200	149	140	NT	NT
18	382139	> 200	> 200	> 200	> 200	NT	NT
19	621109	32	28	36	33	1.4	NR
20	624906	> 200	> 200	> 200	> 200	40	NR
21	642629	> 200	> 200	> 200	> 200	> 100	> 100
22	686564	> 200	> 200	> 200	> 200	78	NR

[a]Values with standard deviation are from three independent experiments, and those separated by semicolons are from two independent experiments.
[b]IC_{50}, 50% inhibitory concentration against purified integrase.
[c]CC_{50}, 50% cytotoxic concentration against uninfected CEM cells.
[d]EC_{50}, 50% effective concentration against HIV-1 infected CEM cells.
[e]NR, not reached due to cytotoxicity.
[f]NT, not tested.
[g]44% protection at 200 μM.

Figure 5(A). The loop in the HIV-1 IN structure[32] used in this study, on the other hand, was disordered and only the residues terminating the loop (i.e., 140 and 148–154) are present in the structure, as shown in Figure 5(B). Of all the crystal structures of HIV-1 IN published to date,[32,51–53] the position of the loop can be unambiguously traced in two structures,[52,53] both of which include the F185H mutation designed to enhance the solubility of HIV-1 IN. The flexible loop adopts very different conformations in those two crystals. In one structure[53] (PDB code 1BL3) the loop is in a conformation similar to that seen in the ASV IN structure, separating the Y3 binding and catalytic sites. However, for the other structure,[52] shown in Figure 5(C) (PDB code 2ITG), the loop assumes an extended conformation protruding away from the Y3 and catalytic sites. This conformation of the loop allows direct access between the Y3 and catalytic sites. In the two structures used in the present work, the presence of the loop in the ASV IN structure separates the Y3 and catalytic sites while the absence of the loop on the HIV IN structure makes the catalytic site accessible from the Y3 site, analogous to the situation in the crystal structure shown in Figure 5(C). Thus, use of both the ASV IN and HIV IN structures for the final docking run effectively allows for two different conformations of the IN flexible loop to be taken into account.

DOCK Selected compounds based on ASV IN

The initial 3100 compounds identified by DOCK using method I showed great diversity. These compounds were then subjected to the more rigorous method II screen. Thirteen (**1, 2, 4, 6–8, 11, 13, 15, 17, 18, 21** and **22**) of the final 22 compounds that were selected for the inhibition study are on the top 200 list of ASV IN. All of them interact with at least one Y3 site residue (Gln-62 and Lys-119) (Fig. 6(A)). Two (**1** and **2**) have tetracycline moieties and both of them interact with Gln-62 and Lys-119 with minimum distances of ca. 3.0 Å. This class of IN inhibitor has been previously identified by a pharmacophore search[25] and has been shown to act on the core domain of HIV-1 IN and to be relatively noncytotoxic. The IN inhibitory assay indicates, however, that only **2** effectively inhibits HIV-1 IN. This may be due to compound **2** being a salt; however, it is more likely that the omission of two hydroxyls on **2** may favor binding, though the mechanism by which this occurs is not clear.

Another group of compounds (**8, 11, 13** and **17**) on the ASV IN list contain benzyl sulfonamide groups which is characteristic of another known class of inhibitors.[18] All these compounds have multiple contacts with residues 146–154 in the flexible loop in addition to their contacts with the Y3 binding residues (Gln-62 and Lys-119). Such interactions may help to fix the IN flexible loop, potentially impacting the binding of DNA (see below). All but one (**17**) are active ($IC_{50} \leq 200$ μM) in both the Mn^{+2} and Mg^{+2} containing solution. Compounds **11** and **13** bind to ASV IN in a similar fashion due to the similarity of their structures, although **13** is in closer contact with both the Y3 site and the flexible loop. Compound **8** has a carboxylate group in addition to its sulfonamide and shows metal-independent activity

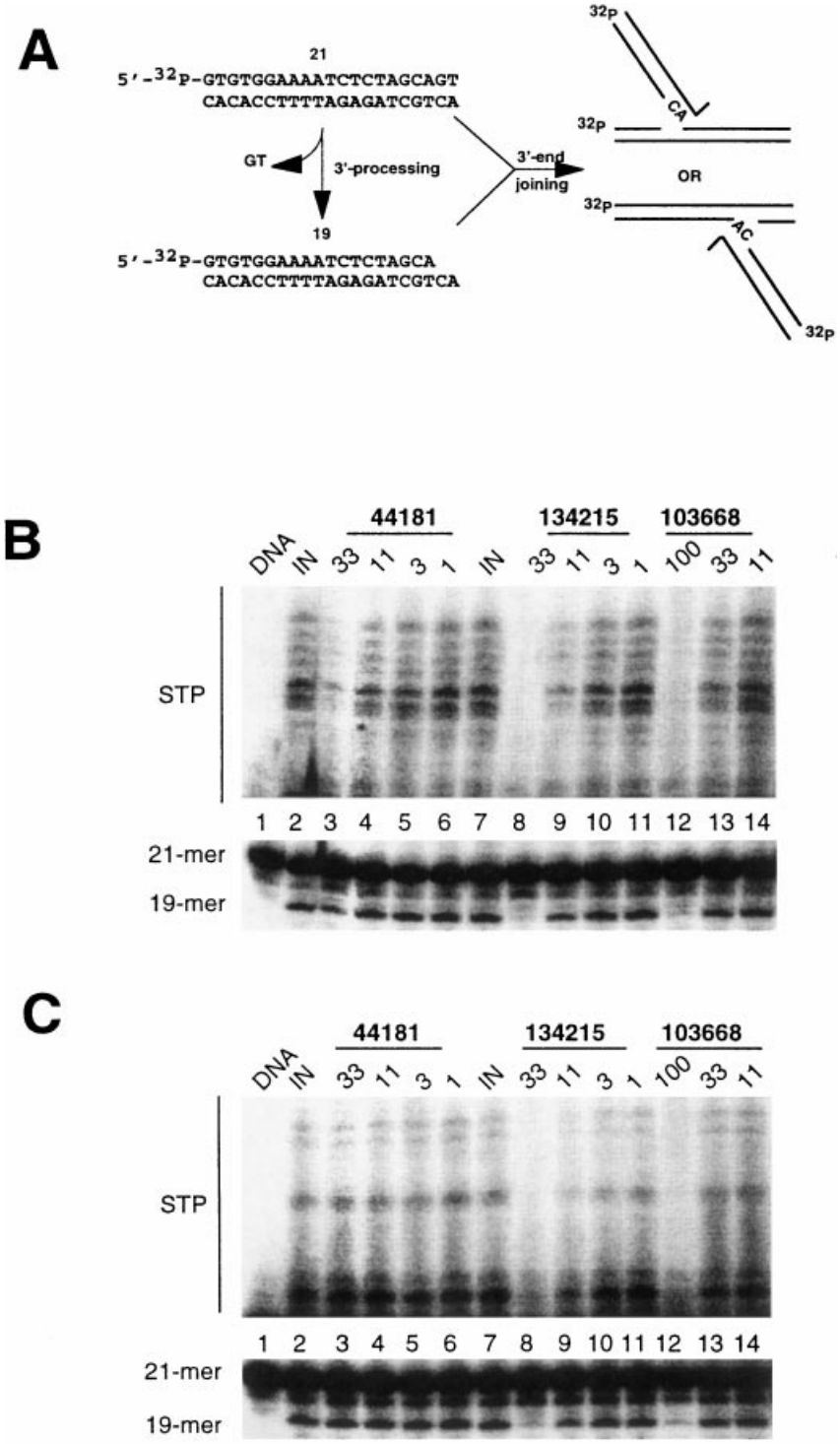

Figure 3. HIV-1 integrase catalytic assays. (A) A 21-mer blunt-end oligonucleotide corresponding to the U5 end of the HIV-1 proviral DNA, 5′ end-labeled with ^{32}P, is reacted with purified HIV-1 integrase. The initial step involves nucleolytic cleavage of two bases from the 3′-end, resulting in a 19-mer oligonucleotide. The second step, 3′-end joining or DNA strand transfer, involves joining of this recessed 3′-end to the 5′-end of an integrase-induced break in another identical oligonucleotide, which serves as the target DNA. (B) Concentration-dependent inhibition of HIV-1 IN by compounds of NSC 44181, 134215 and 103668 (compounds **2, 13,** and **4** in Table 1 and Figure 2) in Mn^{+2} containing reactions. Lane 1 DNA alone; lane 2 DNA plus IN; lanes 3–14 DNA and IN in the presence of indicated concentrations (μg/mL) of inhibitors. (C) Concentration-dependent inhibition of HIV-1 IN by compounds NSC 44181, 134215 and 103668 (compounds **2**, **13**, and **4** in Table 1 and Figure 2) in Mg^{+2} containing reactions. Lane 1 DNA alone; lane 2 DNA plus IN; lanes 3–14 DNA and IN in the presence of indicated concentrations (μg/mL) of inhibitors.

against HIV-1 IN. Analysis of the binding conformation of compound **17** indicates that the bulky part of the molecule may prevent it from binding to the Y3 site, leading to loss of inhibitory activity.

Of the remaining ASV IN selected compounds, compound **7** has direct interactions with Gln-62 (3.2 Å) and Lys-119 (3.1 Å), consistent with the good inhibitory activity. Based on the DOCK-generated binding

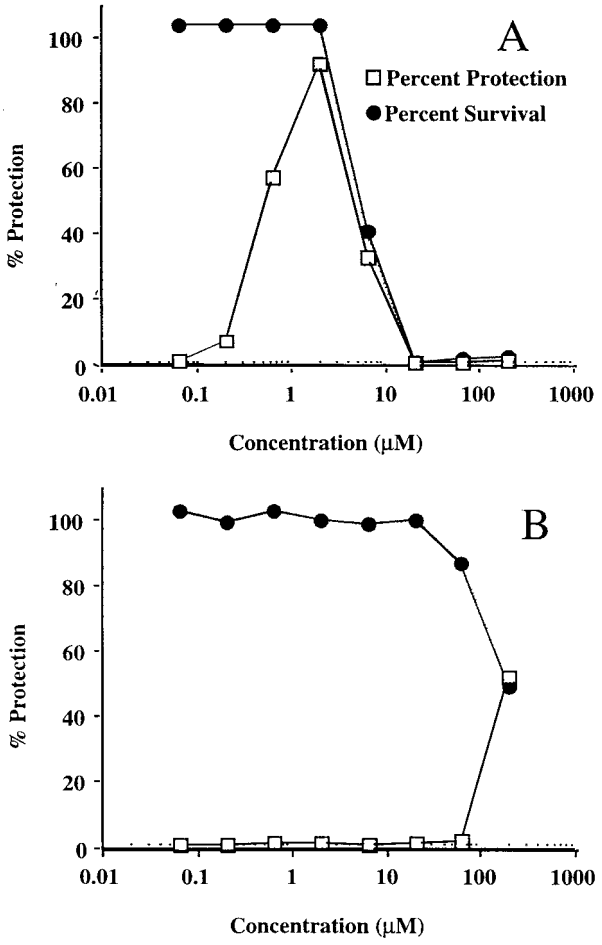

Figure 4. Results of antiviral activity of (A) NSC 97318 and (B) NSC 134135 performed using CEM-SS cells and HIV-1_{RF} using XTT cytoprotection assay as described in the Experimental.

Table 2. C^{α} to C^{α} distances (Å) between residues defining the catalytic site and the Y3 binding site

Y3 site	Catalytic site		
ASV IN	Asp-64	Asp-121	Glu-157
Gln-62	6.9	9.1	9.3
Lys-119	8.9	7.1	12.3
HIV-1 IN	Asp-64	Asp-116	Asp-152
Gln-62	6.9	9.1	10.1
His-114	8.8	7.1	12.8

conformations, compound **15** would be considered as a good candidate to be a strong inhibitor. It has strong interaction with Gln-62 (2.9 Å) and Lys-119 (3.1 Å) and also has multiple contacts with residues 150–154 of the flexible loop. However, it has no inhibitory activity toward HIV-1 IN. Thus, there is no rigorous correlation between the predicted binding interactions with inhibitory activities. In general, the ASV IN selected compounds had interactions with the Y3 binding site and the flexible loop, however, catalytic site contacts were not seen, consistent with the location of flexible loop between the Y3 and catalytic sites (see Figs. 5(A) and 6(A)).

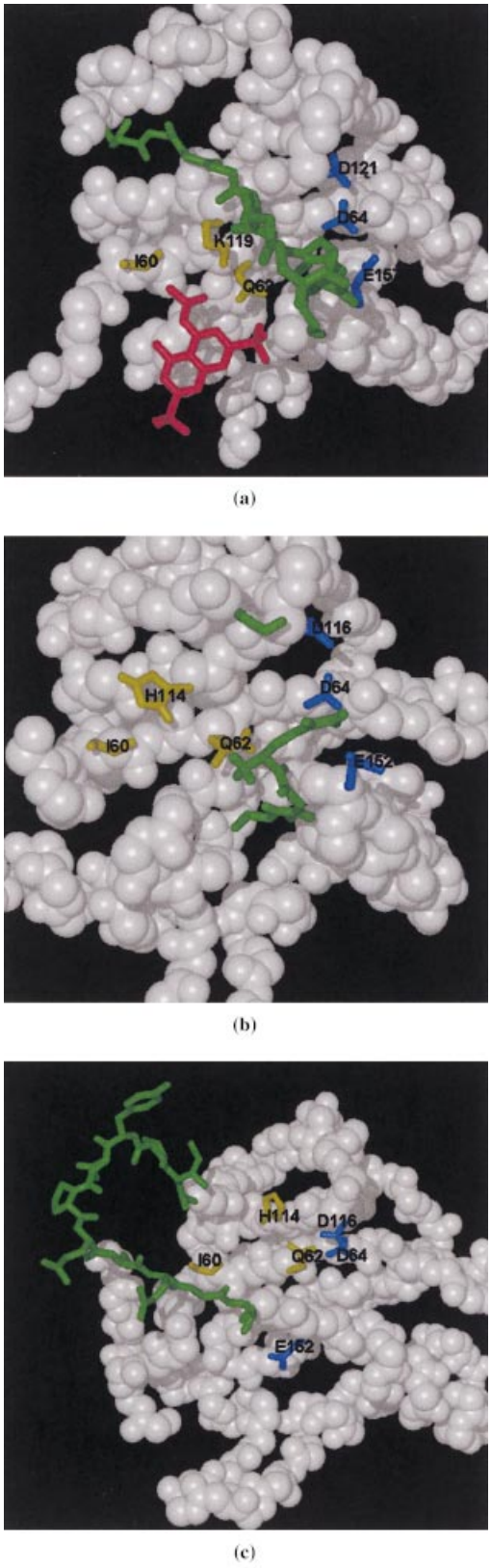

Figure 5. The flexible loop residues (140–154 for ASV IN and 140–150 for HIV IN) are shown in green in (A) ASV IN (1A5V), (B) HIV IN (1BIU), and (C) HIV IN F185H mutant (2ITG) structures. Y3 is shown in red. The Y3 site binding residues are in yellow, and the catalytic site residues are in blue.

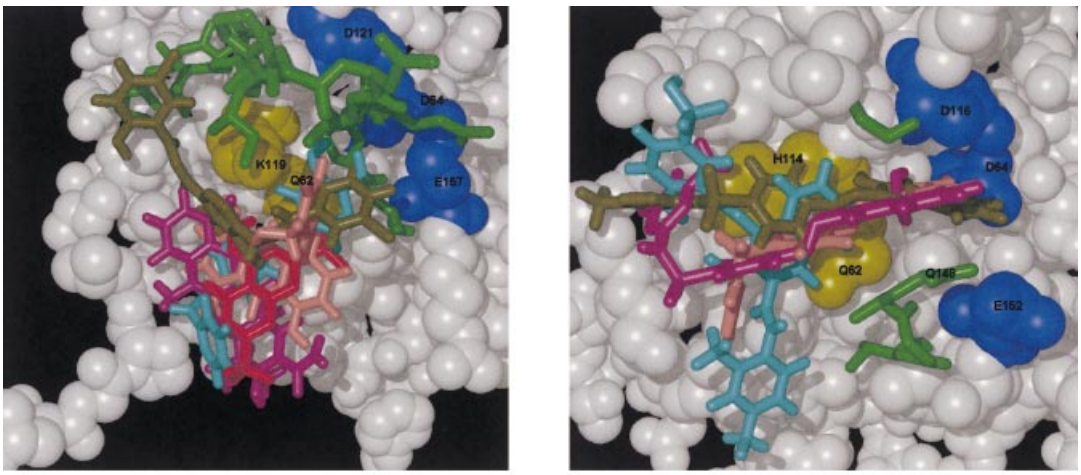

Figure 6. Selected active compounds bound to (A) ASV IN (**2, 4, 7,** and **11**) and to (B) HIV-1 IN (**3, 9, 10,** and **13**). The three catalytic residues (see Table 2) are shown in blue, the two Y3 site residues are in yellow, Y3 is in red, and the flexible loop is in green. For ASV IN bound compounds, **2** is in mauve, **4** is in tan, **7** is in pink, and **11** is in cyan. For HIV IN bound compounds, **3** is in cyan, **9** is in tan, **10** is in mauve, and **13** is in pink.

DOCK Selected compounds based on HIV-1 IN

3100 compounds selected via method II were screened against HIV-1 IN using the method II docking options. Twelve (**3**–**5, 9, 10, 12, 13, 16, 19**–**22**) of the assayed compounds are on the HIV-1 IN top 200 list. Eight of them are active against HIV-1 IN. Interestingly, some of the active compounds from the HIV-1 IN list show an interaction profile with HIV-1 IN involving simultaneous interactions with both the Y3 binding and catalytic sites (Fig. 6(B)). As with ASV IN (Fig. 6(A)) various interactions beyond those with the Y3 and catalytic sites occur for the different inhibitors. In addition, contacts with residues in the flexible loop that are present in the HIV-1 IN structure were observed.

To better investigate interactions with the DD-35-E catalytic site (Asp-64, Asp-116, Glu-152) the eight active compounds ($IC_{50} \leq 200$ μM) from the HIV-1 IN top 200 list were analyzed with respect to their docked binding orientations. Of the 12 HIV-1 IN selected compounds four (**9, 10, 12** and **13**) contain benzyl sulfonamide groups. All benzyl sulfonamide-containing compounds show similar IC_{50} values ($\leq$ 100 μM) and have similar binding modes to HIV-1 IN. The elongated conformation of these compounds enables them to interact simultaneously with Y3 and catalytic site residues. Presented in Figure 7 are schematic diagrams of several of HIV-1 IN selected compounds showing their interactions with Y3 binding site, catalytic site and flexible loop residues. The hydroxyl group of compound **9** interacts with Asp-116, the azo group with Gln-62, and the sulfonamide nitrogen with His-114. Compound **10** has two oxygens of the carboxyl group close to Gln-62 and Asp-116. No catalytic site contact occurs for compound **12**. Compound **13**, the one with the lowest IC_{50} value among all sulfonamide-containing compounds, has interactions with Asp-64 and Asp-116 through two hydroxyl groups on one end of the molecule and with Gln-62 via one of the azo nitrogens more in the center of the molecule. Contacts with Gln-148 of the flexible loop are also seen for all four benzyl sulfonamide compounds. Other effective inhibitors identified by the docking to HIV-1 IN include compounds **3, 4, 5** and **19**. Compound **3** has one ionizable sulfonate group and interacts with the Y3 site and with Gln-148 in the flexible loop. No catalytic site contact is seen for compound **19**, which could explain its metal-independent activity. Three major contacts are also seen for compound **4** to the Y3 and catalytic sites and Gln-148 in the flexible loop. Because of the relatively compact size of compound **5**, it is only in close contact with the Y3 site and the flexible loop (N1 to Gln-62 at 3.1 Å and O1 to Gln-148 at 3.0 Å).

Based on the present results, the majority of active compounds identified from the HIV-1 IN search have simultaneous interactions with Y3 and catalytic site residues. Although the average C^{α} to C^{α} distance between the Y3 and catalytic sites is about 9 Å (Table 2), distances between sidechain atoms of catalytic and Y3 residues (Gln-62 and His-114) can be as short as 5 Å. Also, some of these inhibitors have different affinities toward different metals. While 13 compounds have IC_{50} values below 200 μM in the presence of Mn^{+2}, only eight compounds exhibit IN inhibitory activity in the presence of Mg^{+2}. Interactions of the inhibitors with catalytic site residues may explain why different IC_{50} values are observed in the presence of Mn^{+2} and Mg^{+2}. Thus, the present results suggest that the inhibition of HIV-1 IN by selected compounds may involve interactions with both the Y3 and catalytic sites.

Another important binding feature of a number of the active compounds is their interactions with the flexible loop. The flexible loop has been implicated to bind to the substrate DNA.[33,34,50] As presented above, several of the compounds bind to Gln-148 in HIV-IN. Gln-148 is located at the end of the flexible loop in the HIV IN

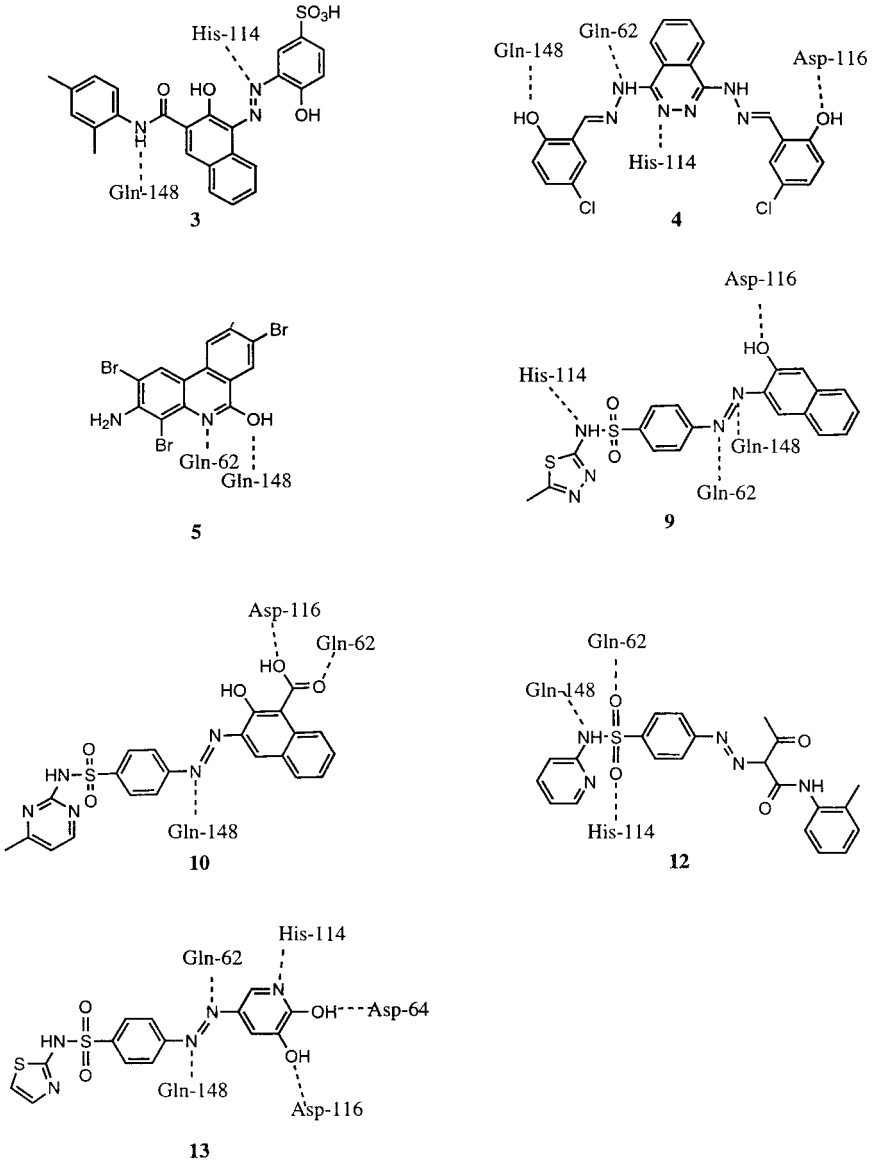

Figure 7. Diagram of interactions between the selected inhibitors and the Y3 binding site residues (Gln-62 and His-114), the flexible loop (Gln-148), and the catalytic site residues (Asp-64, Asp-116, and Gln-152) in HIV-1 IN.

crystal structure and has been shown to be of importance in recognition of viral DNA by a cross-linking experiment.[34] Furthermore, interactions between ASV IN and some of the active compounds occurred via the flexible loop (see above). Since different conformations of the flexible loop occur in different HIV IN crystal structures (see Fig. 4) and also occur between ASV IN alone and the ASV IN-Y3 complex,[30] it is proposed that inhibition by some of the active compounds may be associated with interference of the motion of the flexible loop, thereby altering the interaction between IN and DNA.

Overlap compounds

Compounds common to both the ASV IN and HIV-1 IN lists (up to the top 700 scored compounds in each set) were also collected for bioassays to test whether the selection of the compounds that interact favorably with both enzymes would be a more effective screening method (see following section). Of a total of eight selected overlap compounds, five (**3, 4, 8, 13** and **14**) have IN inhibitory activity and three (**3, 8,** and **13**) showed cytoprotection against HIV-1 infected CEM cells. Among compounds **3, 8,** and **13** the acidic groups (SO_3H and COOH) of **3** and **8** are going to be ionized in the aqueous solution. It is not known, however, whether the ionic state contributes to the antiviral activity. As for the binding orientations to HIV-1 IN, **8** and **13** contact HIV-1 IN in a similar fashion by simultaneously interacting with the Y3 and catalytic sites, while compound **3** interacts solely with the Y3 site of HIV-1 IN. All three compounds that have antiviral activity also interact with Gln-148 of the flexible loop. Additional weak hydrophobic interaction with HIV-1 IN can be found for **8** and **13**. With respect to ASV IN, all these compounds interact only with the Y3 site residues and part of the flexible loop.

Conclusions

HIV-1 IN inhibitors have been identified via a computer based site-directed search of all compounds in the non-proprietary, open part of the NCI 3-D database. Two stages were used to identify potential inhibitors. In the first round, compounds were selected from the entire database by retaining those that had the most favorable interaction energies with the Y3 binding site of ASV IN, yielding a total of 3100 compounds. The preliminary search of the database by method I provided an efficient way to retain compounds with the potential to be good inhibitors. The use of ASV IN as a surrogate target to identify potential HIV-1 IN inhibitor is suggested to enhance the diversity of identified inhibitors and could contribute to the drug design process. Further screening of the 3100 compounds was performed by employing a more rigorous screening approach (method II) against both ASV IN and HIV-1 IN. This yielded 582 compounds, from which 22 were selected for assay as IN inhibitors. Of these nearly 64% had IN inhibitory activity proving that the applied site-directed database search is an efficient way to identify HIV-1 IN inhibitors.

Of the compounds identified in the present study, the majority have structures similar to previously identified HIV IN structures.[17,18] For example, compounds **1** and **2** are tetracyclines[25] and compound **19** is a bis-arylamide.[54] As expected the remaining identified compounds contain a variety of hydrogen bond acceptors and donors, often directly linked to aromatic moieties, which are seen in the majority of known IN inhibitors.[18] While it would be preferable to obtain a larger number of novel compounds, the identification of compounds similar to known IN inhibitors is consistent with the use of the same database as previously used for IN inhibitor identification,[24,55,56] thereby supporting the validity of the applied methodology. While the compounds selected were generally structurally similar to previous identified HIV-1 IN inhibitors, many of them represent novel variations of those compounds that may act as lead compounds for future drug design. Importantly, the most active compound **3** contains novel characteristics not seen in known IN inhibitors, indicating its value as a lead compound. The three lead compounds shown to have antiviral activity (**3**, **8** and **13**) are being tested for further antiviral activities. Studies are also underway to synthesize analogues of these compounds with more desirable biological properties. Results from these studies will be presented elsewhere.

Analysis of DOCK proposed binding orientations was made to see if there is any correlation between IC_{50} values and binding modes. It should be noted that no significant correlation was found between interaction energies and inhibitory activity (not shown). The lack of rigorous correlation may be due, in part, to the simplified potential energy function used to predict and rank the binding orientations. In addition to the electrostatic and van der Waals components included in the present study, there are other important factors that contribute to inhibitor-enzyme binding,[57,58] such as solvation energy, hydrophobicity, and conformational energy. Moreover, the docking approach itself contains simplifications including rigid conformations of the enzyme and limitations in the number of conformations of each small molecule ligand tested. Despite these limitations, general trends regarding contacts between the active inhibitors and ASV IN or HIV-1 IN may give some indications as to the actual binding modes. For active compounds identified from the ASV IN search, all have at least one contact with Y3 binding residues and interactions with the enzyme are generally restricted to the Y3 site region. For active compounds identified from the HIV-1 IN search, most interact simultaneously with the Y3 site and the catalytic site. Many active compounds from both the ASV IN and HIV-1 IN lists are also in contact with part of the flexible loop involved in DNA binding. The unique binding motif involving both the Y3 and catalytic sites identified in the present work could be exploited in the future development of anti-AIDS drugs.

Computational and Experimental Methods

Database searching

Site-directed searching was performed using the program DOCK 4.0.1.[38,43] The database used in this study was the nonproprietary, open portion of the National Cancer Institute (NCI) 3-D database, which consists of 246,182 compounds.[29] 3-D structures of the ASV IN (Protein Data Bank[59] code 1A5V[30]) with the bound disulfonic acid inhibitor, Y3, and the HIV-1 IN with one Mg^{2+} at the active site (code 1BIU[32]) were used in the database searches. All water molecules, the metal cofactors and the bound Y3 were removed prior to docking. Charges and atom types for each compound in the NCI database[60] and for both enzymes were added by the molecular modeling package Sybyl.[61] The solvent-accessible surface[62] of the Y3 binding site on ASV IN was explored via the spheres generated by DOCK. Based on visual inspection, 17 spheres were selected which encompass the Y3 binding site. These spheres were used in the initial screening of the entire database (method I) for the identification of prospective ligands that complement the Y3 binding site. The ligand-IN interaction energies are approximated by the sum of electrostatic and van der Waals components as calculated by the GRID method[41,42] implemented in DOCK. Two important Y3 binding residues (Gln-62 and Lys-119)[30] on ASV IN along with Ile-60 near the Y3 binding site were used to locate the corresponding Y3 binding site on HIV-1 IN. As presented above, sequence and structural alignment of ASV IN and HIV-1 IN indicated that Ile-60, Gln-62 and Lys-119 on ASV IN are equivalent to Ile-60, Gln-62 and His-114 on HIV-1 IN. The coordinates of HIV-1 IN were oriented to minimize the RMS difference between the backbone atoms (C, O, N, C^{α}) of the three corresponding residues on the two enzymes. Y3, based on the coordinates from ASV IN-Y3 complex, was then placed in the HIV-1 IN structure. Visual inspection of the hypothetical HIV-1 IN-Y3 complex showed the absence of any steric clashes and the inhibitor to be adjacent to residues Ile-60, Gln-62

and His-114. This region, therefore, was defined as the Y3 binding site on HIV-1 IN.

Initial screening of the database using method I was done using ASV IN. All compounds were initially selected to contain 10 or less rotatable bonds and between five and 35 nonhydrogen atoms. Ligand flexibility was considered by dividing each compound into a collection of non-overlapping rigid segments. Individual rigid segments with five or more heavy atoms (e.g., aromatic rings) were selected as anchors. Each anchor was docked separately into the binding site in 200 different orientations, based on different overlap of the anchor atoms with the sphere set, and energy minimized. The remainder of each molecule was built onto the anchor in a stepwise fashion, with each step corresponding to a rotatable bond. At each step the dihedral about the rotatable bond was sampled in 10° increments and the lowest energy conformation selected. During the build-up procedure selected conformers are removed based on energetic considerations and maximization of diversity of the conformations being sampled, as previously described,[43,63] with the final selected conformer corresponding to that with the most favorable interaction energy with the binding site. Prior to the search the NCI database was separated into 14 subsets of, on average, 17,600 compounds. The top 200 scored compounds for each subset were stored for later use. Three of these subsets were each further split into two sets of approximately 9000 compounds to further accelerate the calculation. The top 150 compounds from each sub-divided subset were saved, yielding a total of 3100 compounds collected from the initial screening.

A more rigorous docking method, referred to as method II, was used in the secondary screening of the initial 3100 compounds. This approach was used against both ASV IN and HIV-1 IN. Method II included (1) simultaneous energy minimization of the anchor fragment during the stepwise ligand build-up procedure and (2) used more optimized sphere sets. For ASV IN the optimized sphere set was obtained by selecting spheres within 3 Å of all nonhydrogen atoms of Gln-62 and Lys-119 (Y3 binding residues), yielding a total of 7 spheres. For HIV-1 IN the sphere set included those within 5 Å of the Gln-62, His-114 and Y3 nonhydrogen atoms based on the proposed Y3 position derived from the procedure described above. Seven spheres were also obtained for the HIV-1 IN.

Compound selection

The top 200 scored compounds selected using method II against ASV IN, HIV-1 IN and the top 200 compounds that were common to both the ASV IN and HIV-1 IN scored compounds were collected, yielding a total of 582 compounds from the search procedure (18 compounds were present on the top 200 lists of both ASV IN and HIV-1 IN). A subset of these compounds was selected for biological assays based on their availability in the NCI repository, molecular diversity, solubility and potential as drug leads.

Source of assayed compounds

All of the compounds used in this study were obtained from the NCI chemical repository through the Drug Synthesis and Chemistry Branch. Compounds were dissolved in DMSO prior to experiments and the stock solutions were kept at 20 °C.

Spectroscopic analysis

All compounds that were subject to inhibitory assay were also analyzed for purity. NMR spectra were obtained at various temperatures on a Bruker AMX500 operating at 500 MHz for ^{1}H with a triple resonance gradient probe (S:N on ^{1}H~650:1). Samples were dissolved either in DMSO-d_6 or D_2O. One-dimensional ^{1}H spectra at 298 K were acquired with 16 K data points and zero-filled to 32 K with a sweep width of 6024 Hz and a relaxation delay of 2 s. Temperature control was provided by a Eurotherm® VT unit with fluctuations of no more than 0.1 K. Spectra were processed with or without resolution enhancement using Gaussian multiplication (lb = −1, gb = 0.1).

Both positive- and negative-ion fast atom bombardment mass spectra were obtained on a VG 7070E mass spectrometer operated at an accelerating voltage of 6 kV and a resolution of 1500. Glycerol or 3-nitrobenzyl alcohol were used as sample matrices, and ionization was effected by a beam of xenon atoms derived by charge-exchange neutralization of a 1.0–1.2 mA beam of xenon ions accelerated through 8.4–8.9 kV. Spectra were acquired under the control of a VG 11/250 J^+ data system at a scan speed of 10 s/decade, and the matrix background was automatically subtracted. NMR and mass spectra (150 pages) are available as supporting information upon request.

Preparation of oligonucleotide substrates

The HPLC purified oligonucleotides AE117, 5′- ACTGCTAGAGATTTTCCACAC-3′, and AE118, 5′- GTGTGGAAAATCTCTAGCAGT-3′, were purchased from Midland Certified Reagent Company (Midland, TX). The expression system for the wild-type HIV-1 integrase was a generous gift from Drs. T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. To analyze the extent of 3′-processing and 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 to 95 °C, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim, Indianapolis, IN) to separate annealed double-stranded oligonucleotide from unincorporated label.

Integrase assay

Integrase was preincubated at a final concentration of 200 nM with the inhibitor in reaction buffer (50 mM

NaCl, 1 mM HEPES, pH 7.5, 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) at 30 °C for 30 min. Then, 20 nM of the 5′-end ^{32}P-labeled linear oligonucleotide substrate was added, and the incubation was continued for an additional h. Reactions were quenched by the addition of an equal volume (16 μL) of 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, 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:

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

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} values were determined by plotting the drug concentration versus percent inhibition and determining the concentration which produced 50% inhibition.

Anti-HIV assays in CEM cells

The anti-HIV drug testing performed at NCI is based on a protocol described by Weislow et al.[64] In brief, all compounds were dissolved in dimethyl sulfoxide and diluted in 1:100 in cell culture medium. Exponentially growing T4 lymphocytes (CEM cell line) were added at 5000 cells per well. Frozen virus stock solutions were thawed immediately before use, suspended in complete medium to yield the desired multiplicity of infection (MOI≅0.1) and added to the microtiter wells, resulting in a 1:200 final dilution of the compound. Uninfected cells with the compound served as a toxicity control, and infected and uninfected cells without the compound served as basic controls. Cultures were incubated at 37 °C in a 5% CO_2 atmosphere for 6 days. The tetrazolium salt, XTT was added to all wells, and cultures were incubated to allow formazan color development by viable cells. Individual wells were analyzed spectrophotometrically to quantitate formazan production, and in addition were viewed microscopically for detection of viable cells and confirmation of protective activity.

Acknowledgements

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