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Mining the NCI antiviral compounds for HIV-1 integrase inhibitors

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Abstract—HIV-1 integrase (IN) is an essential enzyme for effective viral replication and is a validated target for the development of antiretroviral drugs. Currently, there are no approved drugs targeting this enzyme. In this study, we have identified 11 structurally diverse small-molecule inhibitors of IN. These compounds have been selected by mining the moderately active antiviral molecules from a collection of 90,000 compounds screened by the National Cancer Institute (NCI) Antiviral Program. These compounds, which were screened at the NCI during the past 20 years, resulted in approximately 4000 compounds labeled as 'moderately active.' In our study, chalcone **11** shows the most potent activity with an IC_{50} of 2 ± 1 μM against purified IN in the presence of both Mn^{2+} and Mg^{2+} as cofactors. Docking simulations using the 11 identified inhibitors as a training set have elucidated two unique binding areas within the active site: The first encompasses the conserved D64-D116-E152 motif, while the other involves the flexible loop region formed by amino acid residues 140–149. The tested inhibitors exhibit favorable interactions with important amino acid residues through van der Waals and H-bonding contacts.

1. Introduction

The *pol* gene of HIV encodes for three enzymes, reverse transcriptase (RT), protease (PR), and integrase (IN). Drugs targeting RT and PR have been available for several years.[1,2] HAART (highly active anti-retroviral therapy), the currently used treatment for HIV infection, comprises a drug cocktail of two or more inhibitors to RT and PR.[3–5] This can reduce plasma viral loads below detection limits. However, this sustained reduction in viral load is accompanied by a wide range of adverse effects including metabolic, cardiovascular, renal, immunologic, hematologic, neurologic, and gastrointestinal toxicities.[6] The result of these side effects is often poor patient adherence to the HAART regimen, treatment interruptions, and/or a discontinuation of the therapy all together. These therapeutic outcomes exacerbate the already existing problem of viral drug resistance to HAART components. Viral replication continues in cellular reservoirs during HAART resulting in the selection of drug resistant HIV-1 variant strains. Further complicating the situation, drug resistant mutations within RT and PR often confer cross-resistance to other HAART drug components targeting the same protein.[7,8] The end result is an increase in drug resistant viral loads and disease progression. Unfortunately, once drug resistant viral strains have emerged, present therapeutic options are limited. Studies have also documented the transmission of drug resistant viral strains to newly infected individuals.[9] Considering the complications with the present treatment strategy for HIV-1 infected individuals, new drugs need to be designed targeting different stages of the viral life cycle,[10,11] such as the integration event.

HIV-1 IN is a validated and an important target for the development of novel drugs for several reasons. First, it is an essential enzyme in the retroviral life cycle.[12] Integration of the proviral DNA into transcriptional active sites of the host DNA represents a point of no return.[13] Moreover, a mutation in any of its conserved residues (D64, D116, and E152) reduces the virus' ability to

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replicate. Second, it has no cellular homologue.[14,15] Drugs selectively targeting IN are expected to show low toxicity in the host. Third, the reactions carried out by IN are unique, and sensitive assays exist for testing IN enzymatic activity in vitro.[16] In addition, crystal and NMR structures are available for use in rational structure-based drug design.[17,18] Finally, the combination of IN inhibitors with those directed against PR and RT was shown to be synergistic in several tested models.[19,20] These features make IN well suited for drug design with a low potential for mechanism-based toxicity in humans.

In spite of its obvious importance, IN is a difficult molecular target for the application of structure-based drug discovery. This is due to its shallow substrate binding site located on the surface of the protein, its role in the formation of a multimeric complex in pre-integration complexes, and the lack of a full-length structure for enzyme in the absence or presence of DNA substrate. In the past 10 years, a number of inhibitors have been reported to target IN (for recent reviews, see Refs. 21–27); however, most of them were either cytotoxic or inactive in blocking viral replication. In spite of extensive efforts, only two drugs have been tested thus far in clinical trials.[19,20,28] Interestingly, both compounds were identified through the screening of a large library of synthetic compounds and natural products using purified IN.[19,29]

Recently, we have completed testing of all the compounds, that showed antiviral activities in the NCI drug-screening program, in our enzyme-based assays specific for IN. We previously identified new classes of IN inhibitors by testing selected group of compounds from the NCI screen.[30,31] In the current study, we have evaluated the anti-IN activities of a series of novel small-molecule compounds with molecular weights less than 400. These molecules were selected on the basis of: (1) their structural novelty and demonstrated chemical identity and purity, (2) potential as lead compounds in regard to structural modification and synthesis for future SAR studies, and (3) proven antiviral activity in cell-based assays. In this study, we used these compounds as our training set to perform docking simulations to further explore the possible binding mechanism.

2. Results and discussion

2.1. Compound selection

The National Cancer Institute (NCI) Chemical Database consists of a collection of over half a million unique structures, which have been assembled over the past 45 years from a wide variety of sources.[32] Since little constraint has been placed on the types of structures obtained, this database represents an extraordinarily eclectic collection of organic compounds. The open or publicly available portion of this database contains over 250,000 structures, of which approximately 60% are accessible as actual compounds. This makes the Open NCI Chemical Database the largest freely available, public domain chemical database in existence. A recent comparison of eight large chemical databases has also shown that the Open NCI Database contains by far the greatest number of structures that are unique to it, with approximately 200,000 structures not found in any of the other analyzed databases.[33] The Open NCI Database is thus a unique and valuable resource for testing, for drug development, and for chemical information applications.

Our laboratory has used this database extensively for three-dimensional pharmacophore searching to identify potential compounds for subsequent screening against various molecular targets.[30,31,34] The validity of this approach for identifying new lead compounds for drug development depends on the selected compounds possessing the stated structures. In a targeted analytical evaluation of the Open NCI Chemical Database employing a randomized clustering analysis to generate 298 clusters covering approximately 86% of the database structures, we found by using both NMR and mass spectral analysis that only about 58% of the compounds representing each cluster were the stated structure with an acceptable level of purity (~80% or greater).[35] Thus, it is important that compounds obtained from large chemical libraries, especially those which draw from multiple sources, be characterized early on in the biological evaluation, particularly if structure–activity studies or modeling studies are to be carried out. For the current study we initially selected 36 compounds with proven activity in cell-based assays for further testing on the basis of their structural novelty and sample availability. On the basis of both NMR and mass spectral analysis, 14 of the selected compounds did not correspond to the stated structures. In addition, 6 other compounds contained an unacceptably high level of decomposition products or other contaminants, so they were also eliminated from consideration. Of the 16 compounds with correct structures and acceptable purity, 5 did not fit our criteria of simple and novel structures of $MW \leqslant 400$. The structures of the remaining 11 compounds that are evaluated in this report are given in Table 2.

2.2. Biological activity

Both the cell-based antiviral activities and in vitro enzyme-based results of the 11 leads screened from the moderately active antiviral NCI compounds are listed in Table 1. The approximately 4000 'moderately active' list of compounds was identified by the NCI Drug Screening program during the past 20 years from a collection of 90,000 compounds, which were tested in a six-day cytoprotection assay as described.[49] Chemical structures, predicted physicochemical properties, and docking scores are summarized in Table 2. Flavone **1** is a representative of a growing list of natural products with diverse biological effects that include both anticancer and antiviral activities. For example, some flavonoids were recently reported to have a potent antiviral activity (IC_{50} = 1.6 μg/ml) against herpes simplex type I virus.[36] Compound **1** showed a modest HIV antiviral activity (EC_{50} = 67 μM) with no cytotoxicity at 200 μM. However, the inhibition of both 3′-processing and strand transfer was rather weak (IC_{50} 300–400 μM).

Table 1. Inhibition of IN catalytic activities and HIV antiviral activities of a series of novel small-molecule compounds **1**–**11**

Compound	Inhibition activity[a] (μM)				Antiviral activity[b] (μM)	
	IC_{50} (Mn^{2+})		IC_{50} (Mg^{2+})		CC_{50}	EC_{50}
	3′-proc	ST	3′-proc	ST		
1 (NSC 121858)	390 ± 200	303 ± 100	408 ± 103	499 ± 124	>200	67 ± 30
2 (NSC 57956)	424 ± 129	410 ± 127	138 ± 63	107 ± 60	117 ± 10	3 ± 2
3 (NSC 91877)	380 ± 28	380 ± 28	950 ± 70	950 ± 70	ND	ND
4 (NSC 168719)	579 ± 161	552 ± 130	312 ± 150	296 ± 100	>200	125 ± 63
5 (NSC 137044)	113 ± 5	73 ± 36	180 ± 60	170 ± 50	19 ± 7	4.5 ± 1.3
6 (NSC 197043)	226 ± 46	190 ± 94	481 ± 90	342 ± 113	112 ± 10	39 ± 18
7 (NSC 197049)	116 ± 7	110 ± 10	700 ± 300	550 ± 300	172 ± 35	72 ± 60
8 (NSC 149701)	43 ± 12	32 ± 23	23 ± 9	30 ± 5	>200	132 ± 11
9 (NSC 667263)	25 ± 2	15 ± 6	44 ± 8	36 ± 15	>1000	141 ± 30
10 (NSC 636790)	53 ± 5	47 ± 4	208 ± 13	190 ± 18	450 ± 96	32 ± 12
11 (NSC 660295)	2 ± 1	2 ± 1	2 ± 1	2 ± 1	107 ± 6	23 ± 12

ND, not determined.

[a] 3′-proc, 3′-processing; ST, strand transfer; reported values are means ± SD of at least three independent determinations.

[b] CC_{50}, cytotoxic concentration at 50%; EC_{50}, effective concentration at 50%.

Compounds **2** and **3**, containing a *N*-phenylbenzamide backbone, displayed similar weak potencies for IN inhibition in the presence of Mn^{2+}. However, *N*-phenylbenzamide **2** was nearly 9-fold more potent than **3** in the presence of Mg^{2+}. Interestingly, compound **2** with an EC_{50} of 3 μM was very efficient in blocking viral replication. Thus, the observed weak IN inhibition yet good cellular activity for compound **2** may suggest an interaction with a combination of targets or the formation of a possible reactive metabolite. Compound **4**, used as photographic material with no prior reported biological activity, was found to exhibit moderate antiviral and weak anti-IN activities but with no cytotoxicity at 200 μM.

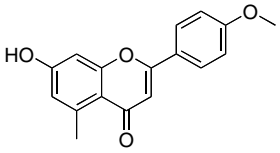
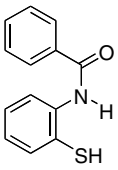
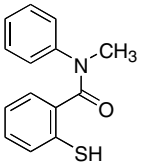
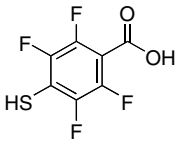
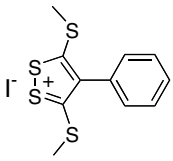
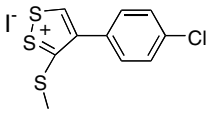
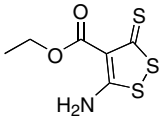
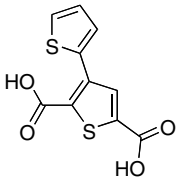
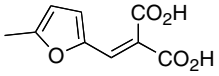
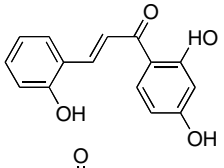
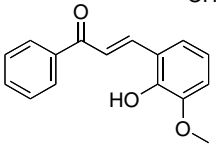
Compounds **5** and **6**, with phenyl-substituted dithionium iodide salts, displayed significantly different activities both in the enzyme-based and the cell-based assays. Compound **5** was at least 2-fold more potent than **6** in all IN assays and 9-fold more potent than **6** in cell-based assays. Compound **7** showed moderate antiviral activity and cytotoxicity with an EC_{50} of 72 μM and CC_{50} of 172 μM, respectively. It showed moderate to weak enzyme inhibition in the presence of Mn^{2+} (IC_{50} = 110 μM for ST) and in the presence of Mg^{2+} (IC_{50} = 550 μM for ST). Compounds **8** and **9**, which are diacids, exhibited a similar range of potencies (IC_{50} values 15–44 μM) for inhibition of IN 3′-processing and strand transfer. Compound **8** was slightly more potent in the presence of Mg^{2+}, while compound **9** showed a better inhibitory effect in the presence of Mn^{2+}. Interestingly, compounds **8** and **9** showed no significant cytotoxicity at the highest tested dose of 200 and 1000 μM, respectively.

The biological activity of chalcones has recently been comprehensively reviewed.[37] This class of compounds exhibits a wide range of anticancer, chemopreventative, antimicrobial, antiviral, and antiprotozoal properties. A variety of chalcones possess cytotoxicity toward a number of different tumor cell lines.[38,39] Several chalcones have demonstrated effective antiviral activities against human rhinovirus, picornaviruses, ECHO 6 virus, and parainfluenza virus. Interestingly, licochalcone inhibited giant cell formation in OKM-1 cells, which were infected with the HIV virus.[37] Compound **10** was approximately 4-fold more active in the presence of Mn^{2+}, inhibiting IN catalytic activities with IC_{50} values of 53 and 47 μM for 3′-processing and strand transfer, respectively. However, compound **11**, which shares the common chalcone motif with **10**, was the most potent compound of all against IN with IC_{50} values of 2 μM for both 3′-processing and strand transfer in the presence of either Mg^{2+} or Mn^{2+}. Moreover, **11** was effective in blocking viral replication with an EC_{50} value of 23 μM and a CC_{50} value of 107 μM. Compound **10** exhibited a similarly effective cellular anti-HIV activity with an EC_{50} of 32 μM but was somewhat less cytotoxic with a CC_{50} value of 450 μM. Therefore, chalcones **10** and **11** are recognized as novel templates for the design of potential inhibitors against IN and viral replication.

2.3. Docking studies

In order to understand the inhibitory mechanism of these 11 effective antiviral compounds, we performed a series of docking simulations. The docking results, in terms of their fitness scores, are summarized in Table 2. The four terms that contribute to the total fitness score are ligand–protein van der Waals interactions, internal ligand van der Waals interactions, ligand–protein H-bonds, and the internal ligand H-bonds. The docking of the 11 compounds yielded fitness score ranging from 32.27 to 49.41. The ligand–protein interactions are shown in Figure 1. We observed two unique binding locations for these 11 compounds (labeled as regions I and II in Fig. 1). Region I is near the active site center defined by the D, D (35)E motif. Region II is close to the active site catalytic loop composed of amino acid residues 140–149. In region I, not only the D, D (35)E conserved motif, but H67, N155, K156, and K159 also interacted through H-bonds or van der Waals interactions with the ligand. Mutations of many of these residues were previously reported to significantly diminish IN enzyme activities thus

Table 2. Structures and properties of the compounds listed in Table 1

Compound	2-D structure	MW	pK_a[a]	clog P[a]	Fitness[b]	Key AA[b]
1		282.29	7.66 (8.3)	3.61 (3.56)	38.76	D116, H77
2		229.30	6.55 (11.80, 3.68)	2.70 (3.06)	41.07	Y143, H114
3		243.32	5.95 (3.53, −1.95)	2.86 (3.29)	37.27	Y143
4		226.15	1.49 (3.94, 1.52)	3.00 (2.74)	36.76	N155, D116
5		398.37	N/A	(5.54)	44.21	H114, N144, Y143
6		386.73	N/A	(5.46)	44.47	Y143, H114
7		221.32	(−2.15)	1.25 (2.32)	32.33	Chelating Mg^{2+}
8		254.28	2.65 (3.48, 2.40)	3.17 (2.9)	49.41	D116,K159
9		196.16	2.13 (4.55, 2.75)	1.78 (1.29)	46.03	D64, H67
10		256.25	7.46 (9.42, 8.39, 7.00)	3.20 (3.32)	46.60	D64, D116, N155, K156
11		254.28	9.18 (8.16)	3.04 (3.66)	42.88	D64, N155

[a] Properties were predicted from Sci-Finder. Values in parentheses were calculated by ADMET Predictor (Simulations Plus, Inc.).
[b] Fitness score was calculated from docking simulations by GOLD. The key amino acid residues were identified involving the ligand binding from docking studies.

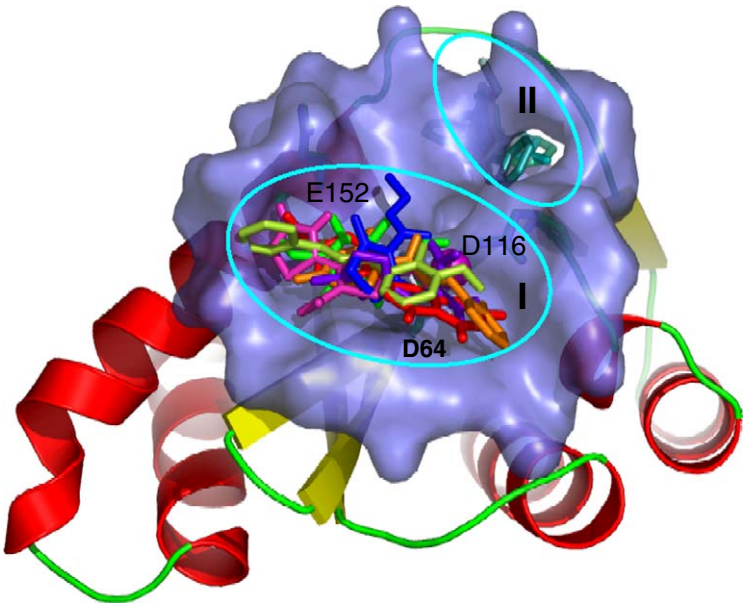


Figure 1. Two unique binding locations were identified from docking simulations, labeled as I and II. Secondary structure of IN is rendered as helices in red, β-sheets in yellow, and loops in green. The magenta sphere below D116 represent Mg^{2+} ion, and stick model represents ligands selected for the docking. Seven compounds (**1**, **4**, and **7**–**11**) dock to region I, and four compounds (**2**, **3**, **5**, and **6**) to region II.

indicating the importance of these amino acid residues for IN activity.[40] Compounds **1**, **4**, and **7**–**11** were clustered in region I (Fig. 1, Table 2).

For the charged molecules, compounds **4**, **8**, and **9**, it is important to take into consideration pK_a values prior to extensive docking studies. When we used both neutral and deprotonated forms in our docking studies of carboxylic compounds, that is, **4**, **8**, and **9**, compounds **4** and **8** resulted in almost identical docked orientation in their neutral versus ionic forms. Only compound **9** adopted an opposite orientation between its two conjugated forms, but docked in the same area labeled as region I.

The remaining compounds, that is, **2**, **3**, **5**, and **6**, were favorably docked in region II (Fig. 1). Amino acid residues Y143, H114, and N144 were recognized as being involved in stabilizing the ligand binding. These three residues showed much less effect on enzymatic activities by previous site-directed mutagenesis studies.[41,42] This may explain their weak potency against IN.

Figure 2A maps the interactions of IN with the representative compound **5** in region II in two-dimensional space. The docking results indicate that compound **5** binds more tightly with IN than **6** by forming an extra H-bond with residue N144. Additionally, H114 not only forms an H-bond interaction with **5**, but hydrophobic interactions were also observed. Moreover, residues I60, Q62, V79, T115, I141, P142, S147, and Q148 were observed to contribute hydrophobic interactions to the favorable docking orientation of compound **5**. This binding mechanism could explain why compound **5** is more effective than **6** in both enzyme inhibition and in exhibiting antiviral behavior. Interestingly, compound **7** did not form any H-bond interactions with IN, but predicted to chelate the Mg^{2+} ion (Fig. 2B). This lack of interaction with IN thus leads to a poor docking score. The predicted binding mode of **10** and **11** (see Figs. 2C and D), rather than their fitness scores, as we discussed earlier, agreed well with their IN inhibition activities. Both chalcones form H-bonds with amino acid residues D64, D116, and N155. However, compound **11** additionally formed hydrophobic interactions with K156, K159, and H67. This binding mechanism may explain the potency of compound **11**.

3. Conclusions

We have identified and confirmed 11 new small-molecule IN inhibitors of diverse structure from a collection of 90,000 tested antiviral compounds in the NCI database. Two different docking locations within the IN structure have been identified that may explain the differential potencies of these lead compounds. Further structural optimization is necessary to develop clinically useful drug candidates.

4. Methods

4.1. Compounds

All compounds were supplied through the NCI Drug Synthesis and Chemistry Branch, Developmental Therapeutics. For biological testing all compounds were dissolved in DMSO and the stock solutions were stored at −20 °C.

4.2. Compound selection and spectroscopic analysis

As explained above, we selected 36 novel small-molecule compounds that exhibited antiviral activity in cell-based

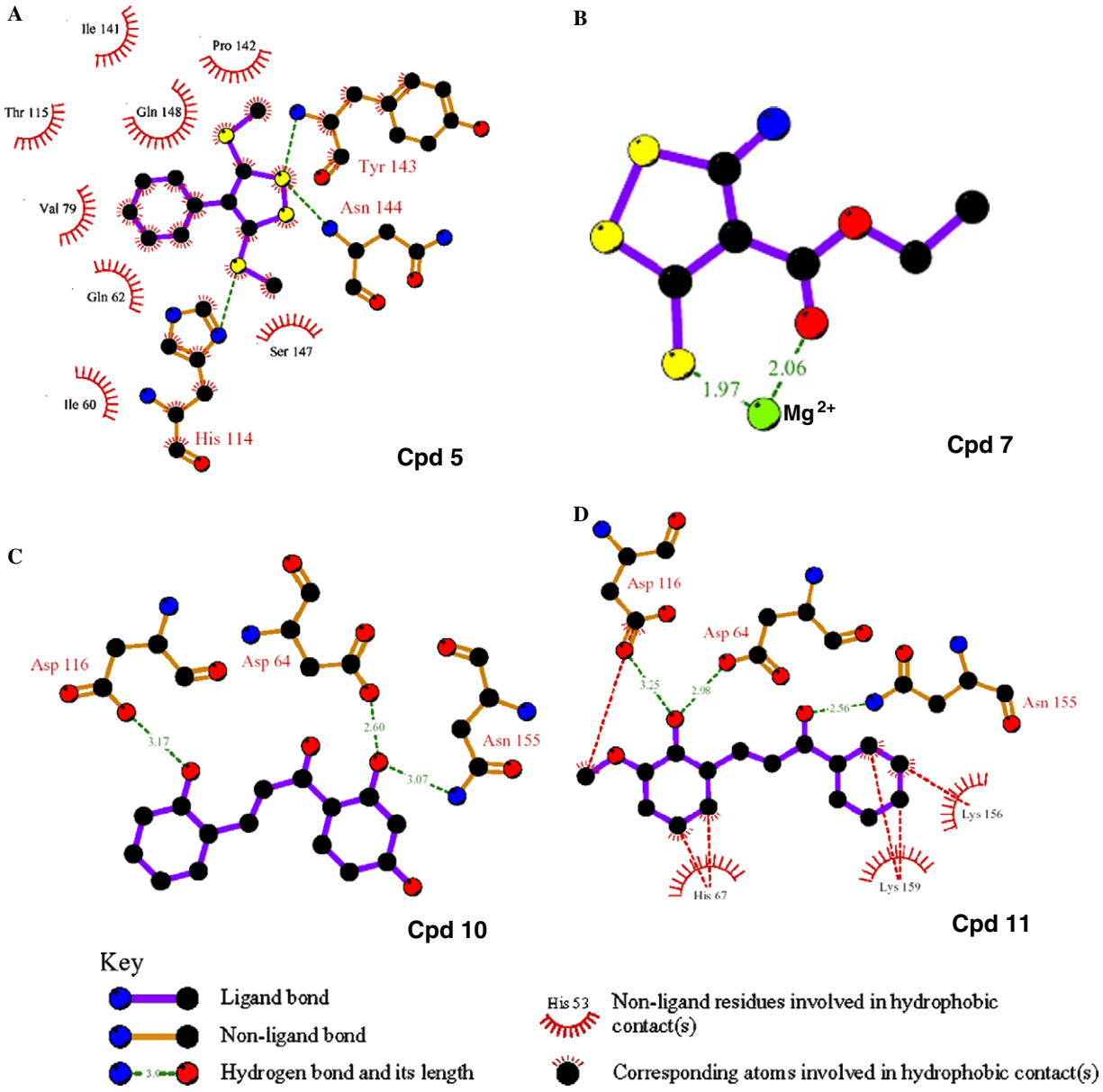


Figure 2. 2-D representations of the ligand–IN interactions predicted from the docking simulations. Ligand–protein interaction maps were generated by Ligplot program.[50]

assays for our in vitro assay specific for IN. All of the compounds evaluated in this study were analyzed to confirm chemical identity and to assess purity. NMR spectra were obtained on a Bruker AMX500 operating at 500 MHz for ^{1}H with a triple resonance gradient probe (S/N on ^{1}H $\sim$ 650:1). Samples were dissolved in either DMSO-d_6 or in D_2O and one-dimensional ^{1}H spectra were acquired at 298 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-HF double-focusing mass spectrometer operated at a scan speed of 10 s/decade and an accelerating voltage of 6 kV under the control of a VG 11/250 J+ data system. Either glycerol or 3-nitrobenzyl alcohol was used as the sample matrix, and ionization was effected by a beam of xenon atoms generated in a saddle-field ion gun at 8.0 ± 0.5 kV. Nominal mass spectra were obtained at a resolution of 1500, and matrix-derived ions were background subtracted during data system processing.

4.3. Docking studies

The docking studies were carried out by the GOLD (version 1.2 Genetic Optimization for Ligand Docking) software package[43] running on our multi-processor linux PC and a 24-processor Silicon Graphics Onyx workstation as described.[44] GOLD has previously been validated and applied successfully to predict the binding orientation of many ligands. We used the crystal

structure of IN bound with the inhibitor 5CITEP (1QS4) complex as the starting structure, and the ligand (5CITEP) was subsequently removed to keep the binding pocket available.[45] Some residues were unresolved in each chain of the X-ray crystal structure. Chain A, which both binds to 5-CITEP, and orients Mg^{2+} chelating D64 and D116, was selected as the docking target. Moreover, the four unresolved residues from chain A were modeled. Y143 and N144 were obtained from chain B of 1QS4, while residues I141 and P142 were modeled from the IN structure 1BIS[46] via backbone alignment.[47,48] All the water molecules present in the crystal structure were removed, but the Mg^{2+} ion was left unaltered. Hydrogen atoms were added to both protein and ligand according to the calculated protonation state at pH 7.0 during the docking studies.

A spherical area with radius of 20 Å was defined and centered at the carboxylate oxygen (OD1) of D64 in the active site. The ligand was then placed within the active site using the least-squares fitting procedure applied in the GOLD program. In order to sample more chemical space, up to 250 conformations of each ligand were generated by Catalyst software within 20 kcal/mol from the minimum. Each conformer was then treated as a separate molecule by GOLD to predict more accurate binding orientations. All docking runs were performed using standard default settings with a population size of 100, a maximum number of 100,000 operations, and a mutation and crossover rate of 95. The scoring function was contributed basically from H-bond interactions, van der Waals interactions within the complex, and the ligand internal energy summarized by ligand steric and torsional energies.[43] Regarding the protonation states of a few compounds containing carboxylic acid, both neutral and ionic forms were selected into the docking studies. For docking fluorine-containing compound **4**, the GOLD parameter file was modified by adding the 'F' atom as an H-bond acceptor to avoid failure initiation of the GOLD program.

4.4. HIV-1 IN inhibition assay

4.4.1. Materials and enzymes. The γ[^{32}P]ATP was purchased from either Amersham Biosciences or ICN. The expression systems for wild-type IN and the soluble mutant $IN^{F185KC280S}$ were generous gifts from Dr. Robert Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD.

4.4.2. Preparation of oligonucleotide substrates. The oligonucleotides 21top, 5′-GTGTGGAAAATCTCTAGC AGT-3′ and 21bot, 5′-ACTGCTAGAGATTTTCCAC AC-3′ were purchased from the Norris Cancer Center Microsequencing Core Facility (University of Southern California) and purified by UV shadowing on polyacrylamide gel. To analyze the extent of 3′-processing and strand transfer using 5′-end labeled substrates, 21top was 5′-end labeled using T_4 polynucleotide kinase (Epicentre, Madison, WI) and γ [^{32}P] ATP (Amersham Biosciences or ICN). The kinase was heat-inactivated and 21bot was added in 1.5-M excess. The mixture was heated at 95 °C, allowed to cool slowly to room temperature, and run through a spin 25 mini-column (USA Scientific) to separate annealed double-stranded oligonucleotide from unincorporated material.

4.4.3. IN assays. To determine the extent of 3′-processing and strand transfer, wild-type IN 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% dimethylsulfoxide, 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 incubation at 30 °C was continued for an additional 1 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, and 0.025% bromophenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M tris-borate, pH 8.3, 2 mM EDTA, 20% acrylamide, and 8 M urea). Gels were dried, exposed in a PhosphorImager cassette, analyzed using a Typhoon 8610 Variable Mode Imager (Amersham Biosciences), and quantitated using ImageQuant 5.2. Percent inhibition (% *I*) was calculated using the following equation:

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

where *C*, *N*, and *D* are the fractions of 21-mer substrate converted to 19-mer (3′-processing product) or strand transfer products for DNA alone, DNA plus IN, and IN plus drug, respectively. The IC_{50} values were determined by plotting the logarithm of drug concentration versus percent inhibition to obtain the concentration that produced 50% inhibition.

4.5. Anti-HIV assays in cultured cells

The anti-HIV activity was evaluated in the human T cell line CEM-SS infected with HIV-1 as described by Weislow et al.[49] In brief, cells were plated in 96-well plates at 5×10^3 cells/well and infected with HIV-1_{RF} (MOI = 0.3). Serial dilutions of compounds were then immediately added to the cells to a final volume of 200 μl. In each experiment, AZT and dextran sulfate were included as control compounds for anti-HIV activity. The cells were maintained at 37 °C with 5% CO_2-containing humidified air for 6 days. Cell viability was quantified by measuring the absorbance at 450 nm after 4 h incubation with 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) at 0.2 mg/ml. Antiviral activity was graded based on the degree of anti-HIV protection as active (80–100% protection), moderate (50–79% protection), and inactive (0–49% protection). Toxicity of the compounds was determined simultaneously on the same plate in uninfected CEM-SS cells.

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