Salicylhydrazine-Containing Inhibitors of HIV-1 Integrase: Implication for a Selective Chelation in the Integrase Active Site

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In previous studies we identified *N*,*N*′-bis(salicylhydrazine) (**1**) as a lead compound against purified recombinant HIV-1 integrase. We have now expanded upon these earlier observations and tested 45 novel hydrazides. Among the compounds tested, 11 derivatives exhibited 50% inhibitory concentrations (IC_{50}) of less than 3 μM. A common feature for activity among these inhibitors is the hydroxyl group of the salicyl moiety. Although the active inhibitors must contain this hydroxyl group, other structural modifications can also influence potency. Removal of this hydroxyl group or replacement with an amino, bromo, fluoro, carboxylic acid, or ethyl ether totally abolished potency against integrase. Several asymmetric structures exhibited similar potency to the symmetric lead inhibitor **1**. The superimposition of the lowest-energy conformations upon one another revealed three sites whose properties appear important for ligand binding. Site A is composed of the 2-hydroxyphenyl, the α-keto, and the hydrazine moieties in a planar conformation. We propose that this site could interact with HIV-1 integrase by chelation of the metal in the integrase active site as inhibition of HIV-1 integrase catalytic activity and DNA binding were strictly Mn^{2+}-dependent. The hydrophobic sites B and C are probably responsible for complementarity of molecular shape between ligand and receptor. Our data indicate that only those compounds which possessed sites A, B, and C in a linear orientation were potent inhibitors of HIV-1 integrase. Although all the active inhibitors possessed considerable cytotoxicity and no apparent antiviral activity in CEM cells, the study presents useful information regarding ligand interaction with HIV-1 integrase protein.

Introduction

The integration of a double-stranded DNA copy of the viral genome into the chromosomal DNA of an infected cell involves two separate events. The first event, known as 3′-processing, occurs after reverse transcription in the cytoplasm of the infected cell. This reaction is a site-specific endonuclease activity that nicks both termini of linear viral DNA adjacent to the invariant CA dinucleotide which is generally located two nucleotides from the 3′-end of the long terminal repeats. The second step, known as 3′-end joining or strand transfer, involves the coordinated nucleophilic attack of the recessed viral 3′-OH on the phosphodiester backbone of the host target DNA.[1,2]

These "cut-and-paste" reactions can be duplicated in an in vitro assay employing HIV integrase, a duplex oligonucleotide corresponding to a viral LTR end, and a divalent metal ion (Mn^{2+} or Mg^{2+}). The expression of integrase in bacteria and subsequent purification to homogeneity allows convenient and rapid isolation of enzyme and identification of inhibitors. Using in vitro assays specific for HIV-1 integrase, numerous classes of inhibitors have been identified, and many of these are polyhydroxylated aromatics particularly in the 1,2-catechol arrangement.[3,4] However, the cytotoxicity of catechols has so far impeded efforts for their development as anti-AIDS agents. More recently, novel non-catechol-based inhibitors of HIV-1 integrase have been discovered.[4] In a recent study, using a three-point pharmacophore, *N*,*N*′-bis(salicylhydrazine) (**1**) was identified as a lead inhibitor of HIV-1 integrase.[5,6] Subsequently, several derivatives were synthesized, and some were shown to be effective inhibitors of HIV-1 integrase in vitro.[6] In an effort to expand upon our early observation, we have searched the NCI Antiviral Drug Screening database and tested 45 novel hydrazides. Herein, we present structure−activity relationship, biochemical, and molecular modeling studies to identify structural requirements for activity.

Results and Discussions

Structure−Activity Relationships. *N*,*N*′-Bis(salicylhydrazine) (**1**) is a potent inhibitor of HIV-1 integrase with IC_{50} values of 2.1 ± 0.8 and 0.7 ± 0.1 μM for 3′-processing and strand transfer, respectively.[6] Table 1 shows that replacement of the hydroxyl group in **1** with amino, bromo, fluoro, ethoxyl, or carboxylic acid group (compounds **2**−**6**) inactivated the compounds. This indicates the importance of the 2′-hydroxyl group. Since neither acidic (COOH) nor basic (NH_2) group substitutions conferred activity, this argues against the simple requirement of hydrogen-bond donor groups for activity.

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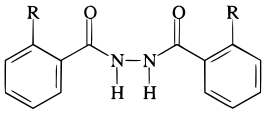
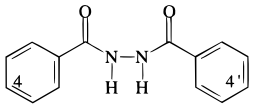
Table 1. Inhibition of HIV-1 Integrase Catalytic Activities and HIV-1 Replication in CEM Cells by a Series of Hydrazides[a]



			IC_{50} (μM)[b]		cellular anti-HIV-1 data (μM)	
compd no.	compound designation	substituent R	3′-processing	strand transfer	IC_{50}	EC_{50}
1	NSC 408200	OH	2.07 ± 0.75	0.73 ± 0.13	0.1	NR[c]
2	NSC 691994	NH_2	>100	>100	>200	>200
3	NSC 88155	Br	>100	>100	NT[d]	NT
4	NSC 88159	F	>100	>100	NT	NT
5	NSC 60386	OCH_2CH_3	>100	>100	NT	NT
6	NSC 157337	COOH	>100	>100	NT	NT

[a] A diagram of the basic structure of the compounds listed is shown above the table. [b] Inhibitory concentration 50%. Values with standard deviations are from three independent experiments. *c*Not reached due to cytotoxicity. [d] Not tested.

Table 2. Inhibition of HIV-1 Integrase Catalytic Activities by a Series of Hydrazides[a]



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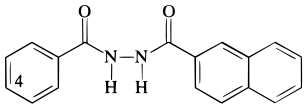

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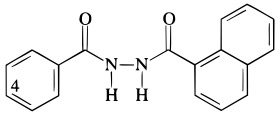


15-17

			IC_{50} (μM)	
compd no.	compound designation	substituent	3′-processing	strand transfer
7	NSC 87963	3,4-$(Cl)_2$	>100	>100
8	NSC 88156	4,4′-$(Br)_2$	>100	>100
9	NSC 91568	3,3′,4,4′-$(Cl)_4$	>100	>100
10	NSC 209076	3,4,5-$(OCH_3)_3$, 4′-Cl	>100	>100
11	NSC 209034	3,4,5-$(OCH_3)_3$, 3′,4′-$(Cl)_2$	>100	>100
12	NSC 88166	2,4-$(Cl)_2$	>100	>100
13	NSC 88168	3,4-$(Cl)_2$	>100	>100
14	NSC 88634	2-F	>100	>100
15	NSC 88167	3,4-$(Cl)_2$	>100	>100
16	NSC 88169	2-Br	>100	>100
17	NSC 88633	2-F	>100	>100

[a] These compounds were not tested for antiviral activity.

Removal of the hydroxyl groups and aromatic ring substitutions (compounds **7**−**17,** Table 2) did not confer activity.

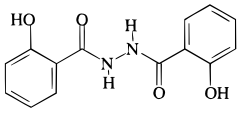
The β-lactam-containing compounds[7,8] exhibited markedly reduced potency against HIV-1 integrase (compounds **18**−**28**, Table 3). Interestingly, substitution of the ancillary phenyl group with either an electron withdrawing or an electron-donating group had no dramatic effect. Addition of an extra ring as in **28** did not change the activity profile of this series of hydrazides (Table 3).

When the hydrazide moiety was restricted in a pyrazolone structure, the anti-HIV-1 integrase activity was essentially dependent on the presence of the 2-hydroxyphenyl group (Table 4). All the compounds containing at least one hydroxyl group[9,10] (**29**−**38**) were potent inhibitors with IC_{50} values for 3′-processing and strand transfer below 3 μM. Substitution of the ancillary phenyl group did not affect the potency. However, removal of the 2-hydroxyl group, as in derivatives **39**−**45**, totally abolished activity. This argues further for the fact that at least one hydroxyl group, next to a carbonyl center, is required for anti-HIV-1 integrase activity.[6]

The antiviral activity of the hydrazides was determined against HIV-1-infected CEM cells and is presented in Tables 1−4. The lead hydrazide **1** exhibited limiting cytotoxicity (IC_{50} value of 0.1 μM), while the diamino compound **2** was nontoxic at 200 μM. However, it did not protect HIV-1-infected cells. Compounds **18**−**28** and **39**−**45** exhibited moderate toxicity in the cell-based assay. Compounds **29**−**38** which exhibited submicromolar potency in the HIV-1 integrase assay were cytotoxic in the nanomolar range. Thus, the most potent integrase inhibitors were generally the most toxic, and the least effective inhibitors were nontoxic in CEM cells.

The issue of cytotoxicity remains very complex and most likely multifactorial. For example, it is well-known that many DNA-binding proteins require a divalent cation (Mn^{2+} or Mg^{2+}) as a cofactor for catalysis. Thus, the cytotoxicity associated with some inhibitors could be metal-dependent. For example, metal complexes of the tetradentate chelating ligand Salen can induce DNA cleavage in the presence of oxidant.[11] These complexes thus act as DNA minor groove binders which could contribute to their nonselective binding. Additionally, the pyridoxal isonicotinoyl hydrazone class of chelators are effective antiproliferative agents.[12]

Molecular Modeling Assessments. The compounds studied were obtained by searching for analogues of the lead compound (**1**) by means of a substructure search in the NCI Drug Information System.[13,14] All of the 45 compounds tested contained the substructure II. Substantial activity against purified HIV-1 integrase (IC_{50} < 3 μM) was found in 11 compounds, and the remaining 34 compounds were inactive (IC_{50} > 100 μM).



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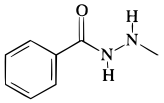
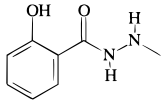


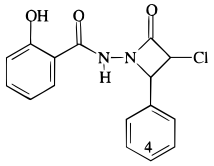
II



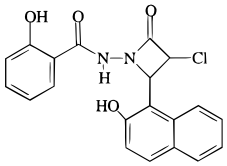
III

Structural analysis of these 45 compounds showed that all the 11 active compounds contained a hydroxyl group attached to the benzene ring at the ortho-position

Table 3. Inhibition of HIV-1 Integrase Catalytic Activities and HIV-1 Replication in CEM Cells by a Series of Hydrazides[a]



18-27

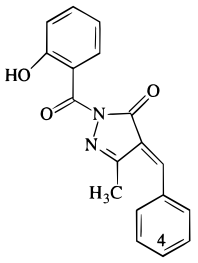


28

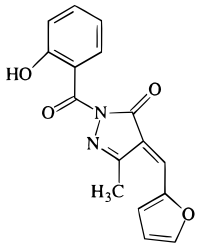
			IC$_{50}$ (μM)[b]					
			3′-processing		strand transfer		cellular anti-HIV-1 data (μM)	
comd no.	compound designation	substituent	expt 1	expt 2	expt 1	expt 2	IC$_{50}$	EC$_{50}$
18	NSC 653030	4-OCH$_3$	288		167		>144	NR[c]
19	NSC 653031	4-NO$_2$	192	168	155	83	>10	NR
20	NSC 653029	2-OH	300		264		8.2	NR
21	NSC 653033	2-Cl	241	248	94		>14.8	NR
22	NSC 653034	3-Cl	155	202	125		>74	NR
23	NSC 653035	4-OH	>300		285		10.3	NR
24	NSC 653036	3-OH	180	225	236	99	>15.0	NR
25	NSC 653037	3-OCH$_3$, 4-OH	>275		262		>27.5	NR
26	NSC 653038	3,4-(OCH$_3$)$_2$	125	109	106		>50	NR
27	NSC 653039	3,4,5-(OCH$_3$)$_3$	68.8	75.7	73.7		4.4	NR
28	NSC 653040		127 ± 42		222 ± 62			

[a] A diagram of the basic structure of the compounds listed is shown above the table. [b] Values with standard deviations are from three independent experiments. *c*Not reached due to cytotoxicity.

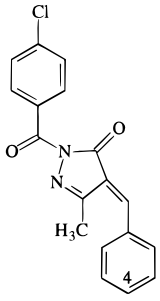
Table 4. Inhibition of HIV-1 Integrase Catalytic Activities and HIV-1 Replication in CEM Cells by a Series of Hydrazides[a]



29-37



38



39-45

			IC$_{50}$ (μM)[b]		cellular anti-HIV-1 data (μM)	
compd no.	compound designation	substituent	3′-processing	strand transfer	IC$_{50}$	EC$_{50}$
29	NSC 652173	2-OH	0.6	2.8	0.062	NR[c]
30	NSC 652174	4-OCH$_3$	0.9	0.6	0.053	NR
31	NSC 652175	4-NO$_2$	0.8	0.6	0.091	NR
32	NSC 652176	3-NO$_2$	1.4	2.6	0.098	NR
33	NSC 652177	4-OH	0.6	0.9	0.059	NR
34	NSC 652178	3-OH	0.9	7.4	0.13	NR
35	NSC 652179	3-OCH$_3$, 4-OH	0.8	0.6	0.094	NR
36	NSC 652180	3,4-(OCH$_3$)$_2$	0.5	0.5	0.15	NR
37	NSC 652182	3,4,5-(OCH$_3$)$_3$	2.0	2.0	0.24	NR
38	NSC 652181		2.7	2.0	0.6	NR
39	NSC 652184	2-OH	>293	>293	>21	>21
40	NSC 652185	3-OH	>293	>293	>8	>8
41	NSC 652186	4-OH	>293	>293	>15	>15
42	NSC 652187	3-NO2	>270	>270	>20	>20
43	NSC 652188	3,4-(OCH$_3$)$_2$	>260	>260	>35	>35
44	NSC 652189	3,4,5-(OCH$_3$)$_3$	>240	>240	>17	>17
45	NSC 652192	4-Cl	>280	>280	>10	>10

[a] A diagram of the basic structure of the compounds listed is shown above the table. [b] Values with standard deviations are from three independent experiments. *c*Not reached due to cytotoxicity.

to the α-keto group. Thus, all the active compounds contained the salicylhydrazine substructure (III).

Eleven out of the 34 inactive compounds tested also contained the salicylhydrazine substructure (III). To understand why these 11 compounds were inactive against HIV-1 integrase, molecular modeling was performed for all the compounds containing the salicylhydrazine moiety. Each structure was built in the ChemNote module of Quanta[15] and energy-minimized using the Adjusted-Basis Newton−Raphson algorithm as implemented in CHARMm.[16] Energy minimization was typically computed with 5000 iterations or until convergence, defined as an energy gradient of 0.001 kcal mol^{-1} Å^{-1} or less. The energy-minimized structures were used as the starting point for the molecular modeling study.

Conformational searches were conducted using the Monte Carlo random search algorithms in QUANTA to find out the lowest-energy conformations of these mol-

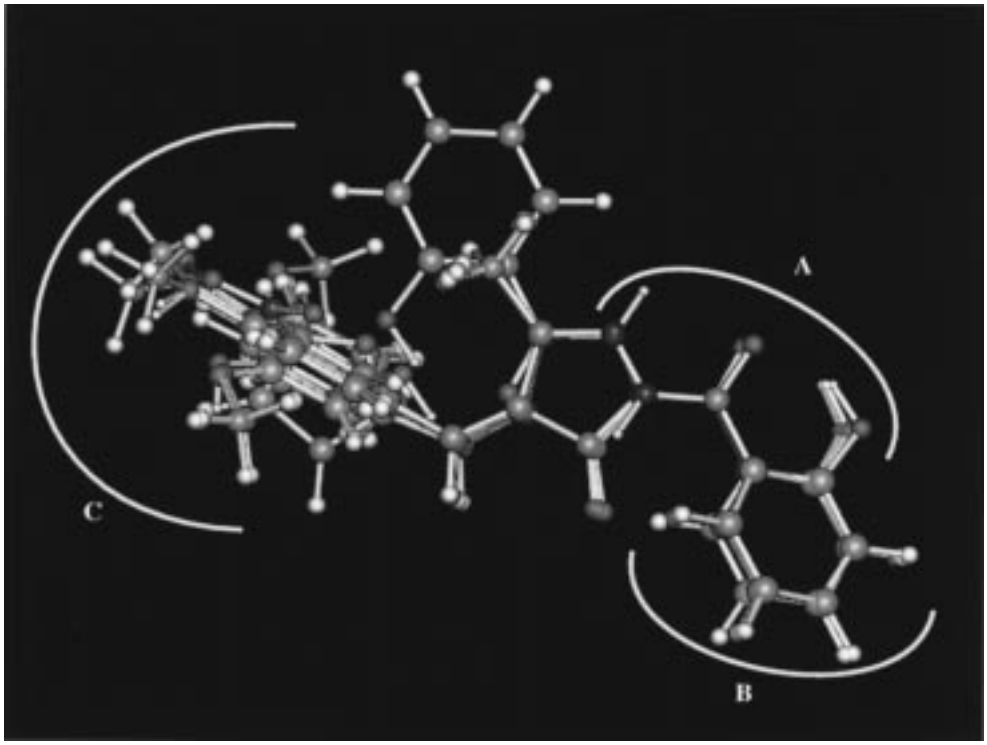


Figure 1. Superimposition of all the active compounds in the "ball-and-stick" model using software Quanta 96. Oxygen, nitrogen, carbon, and hydrogen atoms are shown as red, blue, green, and white balls, respectively, and all the bonds are in white sticks. Site A depicts the metal chelation site or hydrogen-bonding region, site B, the hydrophobic region, and site C, a fingerprint for the complementarity of molecular shape between ligand and enzyme.

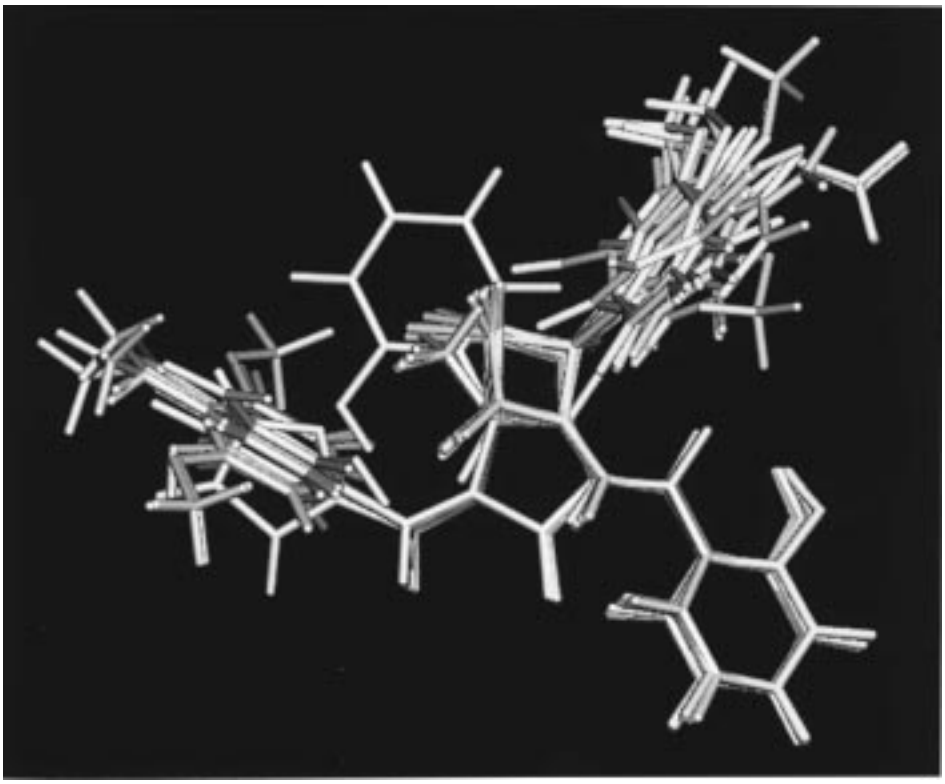


Figure 2. Comparison of the superimposition of the active and inactive compounds. Sites A and B were superimposed for both active and inactive compounds as in Figure 1. Active compounds are shown in white and inactive compounds in yellow.

ecules. In each case, 3000 conformations were generated by random sampling and all were minimized as described above. The superimposition of the lowest-energy conformations upon one another was examined. In this way it was found that all the 11 active compounds could be superimposed upon one another using the salicylhydrazine substructure (III) as shown in Figure 1. Further analysis of Figure 1 suggests three sites whose properties may be important in ligand binding. Site A is one in which electrostatic interac-

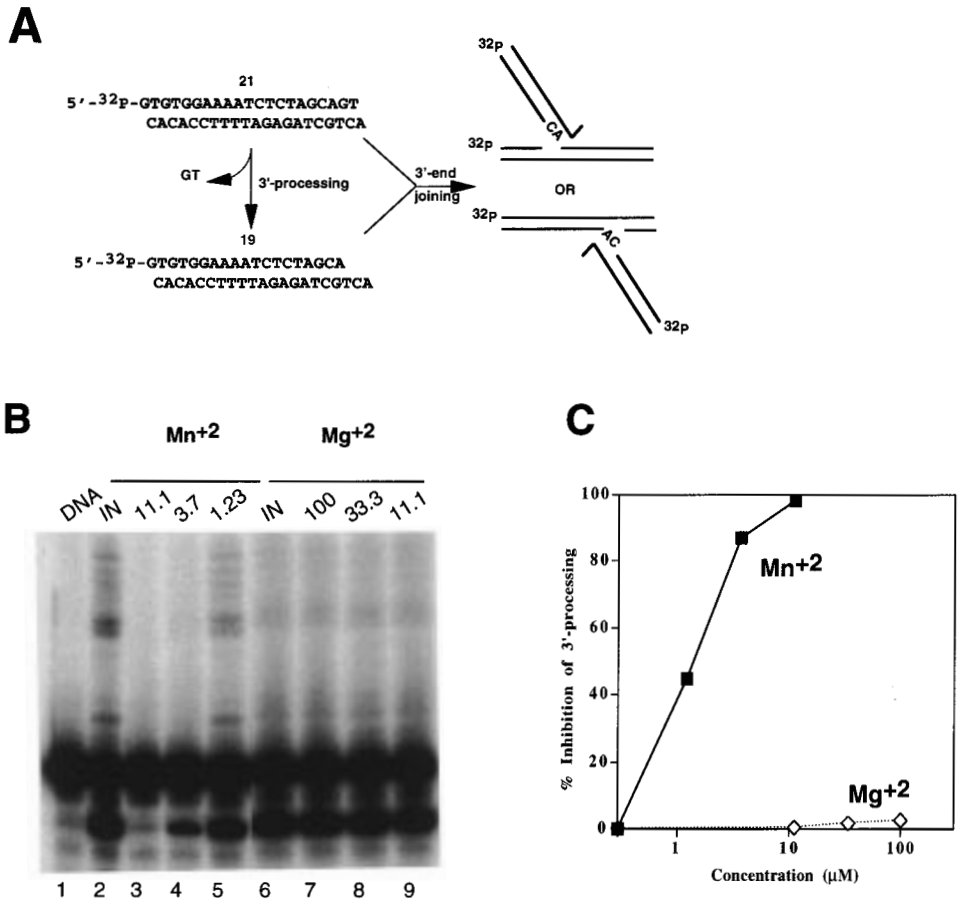


Figure 3. HIV-1 integrase catalytic assays. (A) A 21-mer blunt-end oligonucleotide corresponding to the U5 end of the HIV-1 proviral DNA, 5′-end-labeled with ^{32}P, is reacted with purified HIV-1 integrase. The initial step involves nucleolytic cleavage of two bases from the 3′-end, resulting in a 19-mer oligonucleotide. The second step, 3′-end joining or 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 compound **1**. Lane 1, DNA alone; lanes 2 and 6, DNA plus IN; lanes 3−5, DNA and IN in the presence of indicated concentrations (μM) of drugs and 7.5 mM of $MnCl_2$; lanes 7 and 8, DNA and IN in the presence of indicated concentrations (μM) of drugs and 7.5 mM of $MgCl_2$. (C) Quantitation of the gel in panel B indicating the percent inhibition of the 3′-processing.

tions, especially hydrogen bonding and divalent ion chelation, may be important. Hydrophobic interactions found in sites B and C may be critical for the whole interaction (the complementarity of molecular shape between ligand and receptor). It is of interest that the lowest-energy conformations of the 11 inactive salicylhydrazines can, like the active compounds, be superimposed well using the salicylhydrazine substructure (III) (the yellow part in Figure 2). Though some of the inactive compounds tended to adopt a conformation in which the hydroxyl group was distant from the carbonyl group, the energy difference between these two conformations was very small. Sites A and B were quite similar in both series of active and inactive compounds. However, the orientation of site C differed markedly in the two series. It is tempting to speculate that the folded arrangement of the inactive compounds (yellow in Figure 2) does not permit binding to the integrase active site, while in the case of the active compounds (white in Figure 2), their elongated shape is favorable for binding to HIV-1 integrase.

Role of Divalent Metals. It is well-established that retroviral integrases require divalent metals and that the divalent metal ion probably binds to the highly conserved acidic residues comprising the so-called D,D-(35)E motif.[1,2] Many of the potent HIV-1 integrase inhibitors identified to date contain either a catechol and/or a carboxylic acid group that can chelate metals.[3,4] Therefore, it is possible that the activity of the free acids or the catechol-containing compounds could be due to chelation of the divalent cations at the integrase catalytic site. Although this hypothesis for inhibiting metal-dependent enzymes seems plausible, to the best of our knowledge, there are no mechanistic studies or cocrystal structures available to validate this hypothesis. Therefore, enzymatic assays were performed in the presence of either Mn^{2+} or Mg^{2+} to assess the differential activity of the salicylhydrazide **1** and the role of divalent metals in the inhibition of integrase function. When the 3′-processing and 3′-end joining activities (Figure 3A) were assayed employing Mg^{2+} as a cofactor, the salicylhydrazide **1** was inactive (Figure 3B,C). This compound was inactive even at 1 mM in the presence of Mg^{2+} (data not shown). Thus, the remarkable selectivity for Mn^{2+} over Mg^{2+} suggested intimate interactions of compound **1** with the metal-containing catalytic site or the DDE motif. This observation was further corroborated using a recently described assay[17] (Figure 4A) to trap HIV-1 integrase with its DNA substrate in the presence of divalent metals. Indeed compound **1** inhibited DNA−IN complex formation in the presence of Mn^{2+}, while

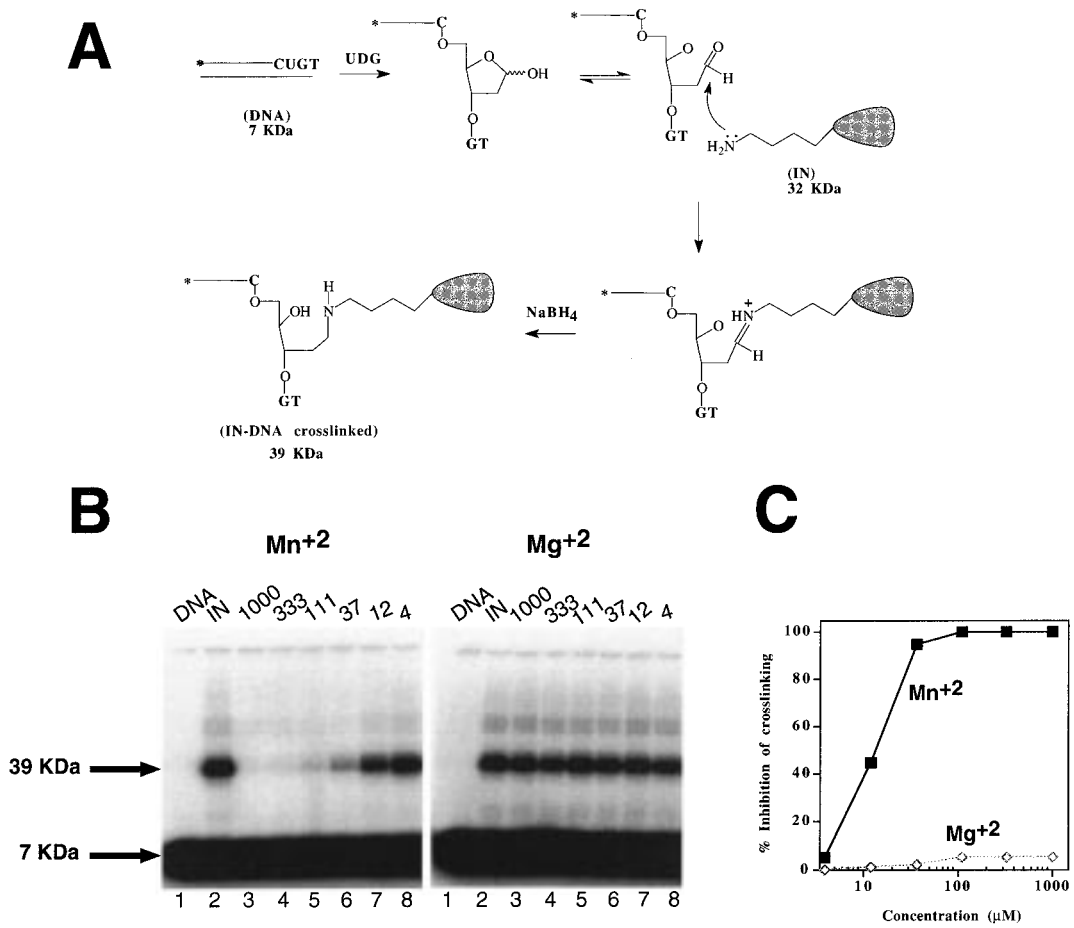


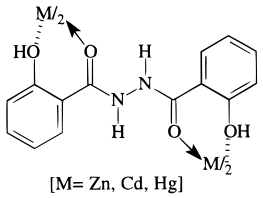
Figure 4. Inhibition of DNA binding of HIV-1 integrase by salicylhydrazine **1**. (A) Principle of the assay.[17] A 21-mer oligonucleotide as described in Figure 3 where the adenine in the conserved CAGT site was replaced with a uracil was 5′-end-labeled. Subsequent treatment with uracil-DNA glycosylase generates an abasic site intermediate which is in equilibrium with its open-form isomer. A nucleophilic attack on the aldehydic sugar residue by lysines of the integrase yields a cross-linked product by forming an imine intermediate which is stabilized by reduction with sodium borohydirde to give a 39-kDa stable cross-linked product. (B) PhosphorImager picture showing the inhibition of 39-kDa DNA−IN cross-linked product in the presence of compound **1** and 7.5 mM manganese chloride (left panel) or 7.5 mM magnesium chloride (right panel). Drug concentrations in micromolar are indicated above lanes. (C) Quantitation of the gels shown in panel B.

no such inhibition was observed with Mg^{2+} at 1 mM drug concentration (Figure 4B).

Two other commercially available compounds that contain an aromatic phenol next to a keto group (piroxicam and niclosamide) were also tested and showed no significant inhibitory potency at 100 μM. Furthermore, a well-know manganese chelator, Salen, and an iron chelator, desferioxamine, were also inactive against purified integrase. These observations further support that chelation per se is not sufficient for inhibition of HIV-1 integrase. In fact, all assays were performed in the presence of excess of divalent metals (7.5 mM of Mn^{2+} or Mg^{2+}). Thus, the requirement of a correct size and a geometric fit in addition to metal chelation seems essential for HIV-1 integrase inhibition.

Other retroviral proteins such as nucleocapsid and reverse transcriptase also require metal ions, and several metal binding sites have been identified on HIV-1 RT.[18] For example, a recent study[19] shows that *N*−(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) inhibits ribonuclease H and DNA polymerase activities of HIV-1 RT. Aromatic disulfides were also reported to eject zinc from the HIV-1 nucleocapsid protein.[20] Therefore, targeting metal binding sites of retroviral enzymes could be potentially exploited as a novel strategy in designing drugs against HIV-1.

It has been reported that the *N*,*N*′-bis(salicylhydrazine) (**1**) can effectively chelate divalent cations as depicted below:[21]



[M= Zn, Cd, Hg]

From the data presented in this study, it seems that the symmetric nature of compound **1** is not necessary and that one side of the molecule is sufficient for inhibition of HIV-1 integrase. Mg^{2+} is more abundant than Mn^{2+} in vivo,[22] and it is generally assumed that the Mg^{2+} is more physiologically relevant for HIV integration. However, a direct proof for selectivity and stoichiometry of HIV-1 integrase for these ions has not been established. Although in many instances Mn^{2+} can substitute for Mg^{2+} in vitro, a question remains whether residual Mn^{2+} can in fact coparticipate in catalysis.

Therefore, selectivity of Mn^{2+}-mediated inhibition of HIV-1 integrase by this class of compound is intriguing.

Thus, the design of a selective inhibitor could utilize the requirement of metals for catalysis by HIV-1 integrase. In conclusion, these types of compounds can be viewed as leads, and identification of water-soluble analogues could be important for cocrystallization with integrase in the presence of metals and for mechanistic studies to elucidate the role and stoichiometry of divalent metals.

Experimental Section

Chemicals. 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, and all aliquots were also made in DMSO prior to each experiment. The stock solutions were kept at −20 °C.

Preparation of Oligonucleotide Substrates. The HPLC-purified oligonucleotides AE117, 5′-ACTGCTAGAGATTTTC-CACAC-3′, AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′, AE157, 5′-GAAAGCGACCGCGCC-3′, and RMAB2, 5′-GTGTGGAAAA-TCTCTAGCUGT-3′, were purchased from Midland Certified Reagent Co. (Midland, TX). The expression system for the wild-type HIV-1 integrase was a generous gift of Drs. T. Jenkins and R. Craigie, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD. To analyze the extents 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 at 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.

To determine the extent of Schiff base formation,[17] RMAB2 was 5′-end-labeled and reacted with AE117 as described above. The uracil was removed from duplex oligonucleotide containing deoxyuridine by incubation of 40 μL of end-labeled DNA (500 nM stock solution) with 1 unit of uracil-DNA glycosylase (Life Technologies, Inc.) for 90 min at 30 °C. The reaction mixture was then loaded on a G-25 Sephadex quick spin column to remove the unincorporated label and the uracil.

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 incubation 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, 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:

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

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 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.

DNA Binding Assay Using Schiff Base Formation. This assay has recently been described in detail.[17] Briefly, IN (200 nM) was preincubated with the inhibitor (at the indicated concentration) for 30 min at 30 °C. Subsequently, an oligonucleotide containing an abasic site[17] in reaction buffer as described above was added for 2 min at room temperature. A freshly prepared solution of sodium borohydride (0.1 M final concentration) was added, and reaction was continued for an additional 2 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. Gels were analyzed using a Molecular Dynamics PhosphorImager. The magnesium-based assays were carried in the presence of 5% PEG as described.[23]

Anti-HIV Assays in Cultured Cell Lines. The anti-HIV drug testing performed at NCI is based on a protocol described by Weislow et al.[24] 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/well. Frozen virus stock solutions were thawed immediately before use, suspended in complete medium to yield the desired multiplicity of infection (m.o.i. ~ 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.

Molecular Modeling. All molecular modeling studies were performed with the QUANTA 4.0/CHARMm22 (Molecular Simulations, Burlington, MA)[15] molecular modeling package running on a Silicon Graphics IRIS Indigo workstation. The energy minimization was typically computed with 5000 iterations or until convergence (defined as an energy gradient of 0.001 kcal mol^{-1} Å^{-1} or less), using Adjusted Basis Newton−Raphson algorithm as implemented in CHARMm.[16] The structures of compounds were built using the ChemNote module with QUANTA and were energy-minimized using CHARMm. Conformational searches were conducted using the Monte Carlo random search algorithms implemented in QUANTA to find the lowest-energy conformations for each compound. The analogues of lead compounds were retrieved using a substructure search of NCI DIS system.[13,14]

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References

(1) Katz, R. A.; Skalka, A. M. The retroviral enzymes. *Annu. Rev. Biochem.* **1994**, *63*, 133−173.
(2) Rice, P.; Craigie, R.; Davies, D. R. Retroviral integrases and their cousins. *Curr. Opin. Struct. Biol.* **1996**, *6*, 76−83.
(3) Pommier, Y.; Pilon, A.; Bajaj, K.; Mazumder, A.; Neamati, N. HIV-1 integrase as a target for antiviral drugs. *Antiviral Chem. Chemother.* **1997**, *8*, 483−503.
(4) Neamati, N.; Sunder, S.; Pommier, Y. Design and discovery of HIV-1 integrase inhibitors. *Drug Discovery Today* **1997**, *2*, 487−498.
(5) Hong, H.; Neamati, N.; Wang, S.; Nicklaus, M. C.; Mazumder, A.; Pommier, Y.; Zhao, H.; Burke, J. T. R.; Milne, G. W. A. Discovery of HIV-1 integrase inhibitors by pharmacophore searching. *J. Med. Chem.* **1997**, *40*, 930−936.
(6) Zhao, H.; Neamati, N.; Sunder, S.; Hong, H.; Wang, S.; Milne, G. W. A.; Pommier, Y.; Burke, T. R., Jr. Hydrazide-containing inhibitors of HIV-1 integrase. *J. Med. Chem.* **1997**, *40*, 937−941.

(7) Singh, I. P.; Gurtu, S.; Kumar, A.; Sinha, J. N.; Bhargava, K. P.; Shankar, K. Antiinflammatory activities of compounds derived from salicylic and benzoic acids. *Arch. Pharm. (Weinheim, Ger.)* **1984**, *317*, 609−614.
(8) Tandon, M.; Kumar, P.; Bhalla, T. N.; Bhargava, K. P.; Barthwal, J. P. Some new azetidinones as possible antiinflammatory, analgesic and antiproteolytic agents. *Acta Pharm. Jugosl.* **1983**, *33*, 93−102.
(9) Pathak, R. B.; Bahel, S. C. Synthesis of some N1-substituted-3,5-dimethyl pyrazoles and N1-substituted-3-methyl-5-pyrazolones and related compounds as potential fungicides. *J. Indian Chem. Soc.* **1980**, *57*, 1108−1111.
(10) Hogale, M. B.; Pawar, B. N. Synthesis and biological activity of N1-substituted-3- methyl-5-pyrazolones and related compounds. *J. Indian Chem. Soc.* **1989**, *66*, 135−138.
(11) Gravert, D. J.; Griffin, J. H. Specific DNA cleavage mediated by [SalenMn(III)]+. *J. Org. Chem.* **1993**, *58*, 820−822.
(12) Richardson, D. R.; Tran, E. H.; Ponka, P. The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* **1995**, *86*, 4295−4306.
(13) Milne, G. W. A.; Nicklaus, M. C.; Driscoll, J. S.; Wang, S.; Zaharevitz, D. W. The NCI drug information system 3D database. *J. Chem. Inf. Comput. Sci.* **1994**, *34* (4), 1219−1224.
(14) Milne, G. W. A.; Miller, J. A. The NCI drug information system. *J. Chem. Inf. Comput. Sci.* **1986**, *26*, 154−197.
(15) QUANTA/CHARMm, amms, is supplied by Molecular Simulations Inc., 16 New England Executive Park, Burlington, MA 01803.
(16) Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. CHARMm: A program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* **1983**, *4*, 187−217.
(17) Mazumder, A.; Neamati, N.; Pilon, A. A.; Sunder, S.; Pommier, Y. Chemical trapping of ternary complexes of human immunodeficiency virus type 1 integrase, divalent metal, and DNA substrates containing an abasic site. Implications for the role of lysine 136 in DNA binding. *J. Biol. Chem.* **1996**, *271*, 27330−27338.
(18) Davies, J. F.; Hostomska, Z.; Hostomsky, Z.; Jordan, S. R.; Mathews, D. A. Crystal structure of the ribonuclease H domain of HIV-1 reverse transcriptase. *Science* **1991**, *252*, 88−95.
(19) Borkow, G.; Fletcher, R. S.; Barnard, J.; Arion, D.; Motakis, D.; Dmitrienko, G. I.; Parniak, M. A. Inhibition of the ribonuclease H and DNA polymerase activities of HIV-1 reverse transcriptase by N-(4-*tert*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone. *Biochemistry* **1997**, *36*, 3179−3185.
(20) Rice, W. G.; Turpin, J. A. Virus-encoded zinc fingers as targets for antiviral chemotherapy. *Rev. Med. Virol.* **1996**, *6*, 187−199.
(21) Tewari, S. R.; Bajpai, S. K.; Misras, S. K.; Shukla, G. N.; Misra, V. N. Inorganic corrdination complexes. Part-V. Complexes of divalent zinc, cadmium and mercury with substituted salicyl hydrazines. *J. Indian Chem. Soc.* **1983**, *59*, 891−892.
(22) Cowan, J. A. *The Biological Chemistry of Magnesium*; VCH: New York, 1995.
(23) Engelman, A.; Craigie, R. Efficient magnesium-dependent human immunodeficiency virus type 1 integrase activity. *J. Virol.* **1995**, *69*, 5908−5911.
(24) Weislow, O. W.; Kiser, R.; Fine, D.; Bader, J.; Shoemaker, R. H.; Boyd, M. R. New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS antiviral activity. *J. Natl. Cancer Inst.* **1989**, *81*, 577−586.

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