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A novel mechanism for inhibition of HIV-1 reverse transcriptase☆

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Abstract

The human immunodeficiency virus (HIV) epidemic is an important medical problem. Although combination drug regimens have produced dramatic decreases in viral load, current therapies do not provide a cure for HIV infection. We have used structure-based design and combinatorial medicinal chemistry to identify potent and selective HIV-1 reverse transcriptase (RT) inhibitors that may work by a mechanism distinct from that of current HIV drugs. The most potent of these compounds (compound **4**, 2-naphthalenesulfonic acid, 4-hydroxy-7-[[[[5-hydroxy-6-[(4-cinnamylphenyl)azo]-7-sulfo-2-naphthalenyl]amino]carbonyl]amino]-3-[(4-cinnamylphenyl)-azo], disodium salt) has an IC_{50} of 90 nM for inhibition of polymerase chain extension, a K_d of 40 nM for inhibition of DNA–RT binding, and an IC_{50} of 25–100 nM for inhibition of RNaseH cleavage. The parent compound (**1**) was as effective against 10 nucleoside and non-nucleoside resistant HIV-1 RT mutants as it was against the wild-type enzyme. Compound **4** inhibited HIV-1 RT and murine leukemia virus (MLV) RT, but it did not inhibit T_4 DNA polymerase, T_7 DNA polymerase, or the Klenow fragment at concentrations up to 200 nM. Finally, compound **4** protected cells from HIV-1 infection at a concentration more than 40 times lower than the concentration at which it caused cellular toxicity.

1. Introduction

Despite a worldwide effort, human immunodeficiency virus (HIV)[6] infection and the subsequent acquired immunodeficiency syndrome (AIDS) remain important medical problems. Although the recent dramatic drop in the AIDS mortality rate and opportunistic infections in the United States is a tribute to new drug therapies [1], the number of people infected by HIV continues to increase [2]. Current treatment of HIV infection in the United States commonly involves three drugs: two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease inhibitor (PI) [3]. These combination regimens can dramatically decrease viral load [3]; however, despite these improvements, replication-competent HIV can still be recovered from patients after 2 years of successful therapy [4].

Resistance has limited the prolonged efficacy of all HIV drugs so far developed. HIV replication generates on average approximately one mutation each time the genome is copied [5]. This genomic diversity makes development of resistant strains almost certain under conditions of selective (drug) pressure, high viral load, and rapid replication. Hecht et al. [6] recently reported the transmission of an HIV strain

[6] *Abbreviations used:* HIV, human immunodeficiency virus; RT, reverse transcriptase; RNaseH, ribonuclease H; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

which was resistant to four protease inhibitors as well as two reverse transcriptase inhibitors. The emergence of multi-drug resistant strains of HIV demonstrates the need for the development of new drugs whose mechanism of action is distinct from those of known drugs.

Reverse transcriptase, an essential enzyme for HIV replication [7], has two enzymatic functions, the polymerase and the ribonuclease H (RNaseH). In the normal viral replication cycle, RT converts viral genomic RNA into a double stranded linear DNA. The success of both NRTIs and non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the treatment of HIV infection demonstrates that HIV-1 RT is a valid drug target.

All HIV-1 RT inhibitors which are either approved or in clinical trials bind to either the NRTI binding site or the NNRTI binding site [8,9]. Several other classes of RT inhibitors have been identified including dyes, natural products, sulfated polysaccharides, and small naphthalene sulfonic acids [10–12]. Some of these compounds specifically inhibit HIV-1 RT, while others inhibit a wide variety of polymerases [13–16]. The mechanism of inhibition has not been established for most of these compounds. However, in gel-shift studies by Hizi and co-workers [15,17], a series of marine natural products appears to block RT–nucleic acid binding and RT polymerase activity, but paradoxically, *these compounds do not inhibit* RNaseH activity. In this paper, we use structure-based design, molecular similarity, and combinatorial medicinal chemistry to identify and develop a chemically distinct class of compounds that inhibit the nucleic acid binding, RNaseH activity, and polymerase activity of HIV-1 RT with low-nanomolar potency (Fig. 1).

2. Materials and methods

Structure-based design. The DOCK algorithm has been described in detail elsewhere [18–21]. As a brief overview, all small-molecule-sized concavities in the molecular surface of a macromolecule are filled with atom-sized spheres to generate a negative image of each potential binding site. Distances between sphere centers are matched to distances between ligand atoms to produce thousands of potential orientations of each ligand within the site. Each ligand orientation is evaluated based on steric and chemical complementarity to the target macromolecule in order to create a list of the molecules predicted to bind to the target.

To identify potential binding sites, we calculated a molecular surface [22] of the RT–DNA complex [23] and generated 29 clusters of spheres. Three clusters (e.g., binding sites) were selected based on their distance from the NNRTI binding site, functional significance, sequence conservation of nearby residues, crystallographic reliability, and geometric quality of the pocket.

A database of commercially available small molecules was prepared from the ACD v93.2 (Available Chemicals Directory, Molecular Design, San Leandro, CA). Three-dimensional structures of each compound were obtained using CONCORD [24] and partial charges were generated using the Gasteiger–Marsili [25] method.

Compounds were evaluated using the intermolecular van der Waals and electrostatic terms from the AMBER force-field [26] as well as an empirical scoring

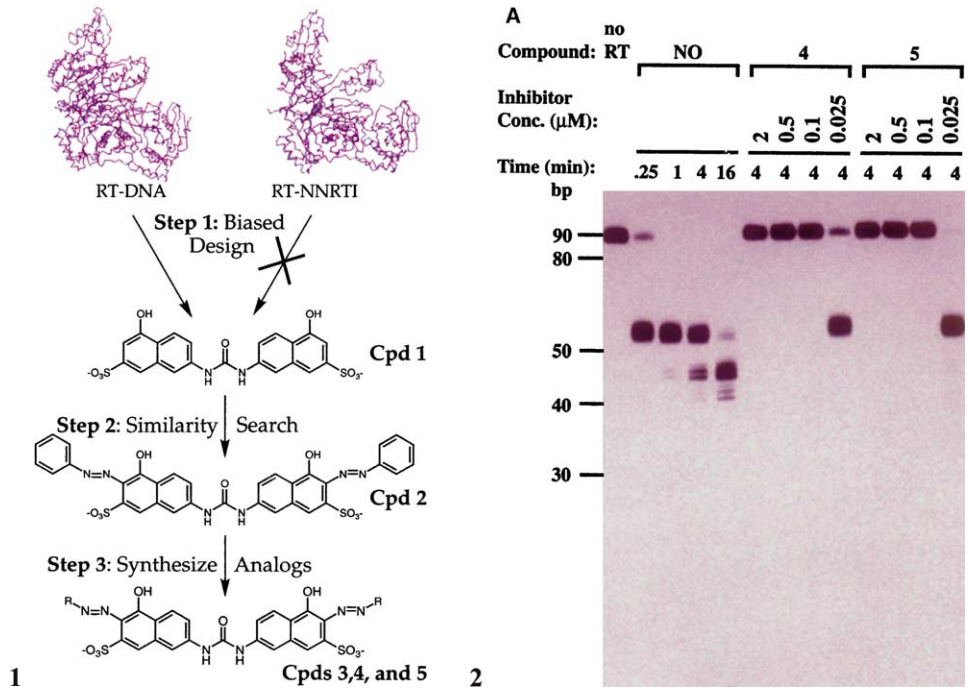


Fig. 1. Ligand design and optimization. A biased structure-based design scheme (step 1) was used to identify initial inhibitors. Similar commercially available compounds were purchased and assayed (step 2). A series of analogs were synthesized, which were potent and selective inhibitors of HIV-1 RT–DNA binding, and thus, polymerase and ribonuclease activity.

Fig. 2. RNaseH Inhibition. HIV-1 RT cleavage of the RNA strand of an RNA–DNA duplex is completely inhibited at concentrations of 2, 0.5, and 0.1 μM and partially inhibited at 0.025 μM by compounds **4** and **5** (A).

function (Ewing and Kuntz, unpublished results). Compounds were selected for biological screening based on: (i) force-field and empiric score (ii) dissimilarity to known non-nucleoside inhibitors, (iii) visual inspection of their proposed binding mode, (iv) solubility and reactivity, (v) chemical diversity, (vi) toxicity, and (vii) ease of analog synthesis. Dissimilarity was assessed using the connectivity metric described by Bemis [27].

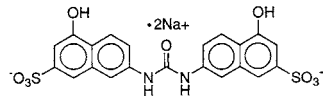
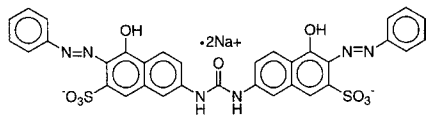
Similarity searches. The ACD v95.1 (Available Chemicals Directory, Molecular Design, San Leandro, CA) was converted to Daylight format [29]. Searches were carried out with Daylight's connectivity measure of similarity and the Tanimoto similarity metric [30]. Daylight's Merlin search engine [29] was used to probe the ACD for related structures.

Reagent selection. To assist in the design of combinatorial analog libraries, a new program, UC_Select [31], was used to identify reagents from the ACD that were both chemically compatible with analog synthesis (vida infra) and had appropriate medicinal properties (i.e., solubility, molecular weight, non-toxicity, and non-reactivity).

Chemicals. Compound **1** was purchased from Pfaltz and Bauer (Waterbury, CT). Compound **2** was purchased from Sigma. While the ACD structure for **2** is asymmetric, we found that the correct structure is the symmetric compound shown in Table 1 (based on mass spectrum, NMR, and HPLC characterization, data not shown). All other chemicals were purchased from Acros and Aldrich and used without further purification.

General. L-SIMS and electrospray mass spectral analyses were performed by the UCSF mass spectrometry facility, A.L. Burlingame, Director. MALDI spectra were performed on a PerSeptive Biosystems Voyager-DE instrument and internally

Table 1
HIV-1 RT binding and inhibition[a]

No.	Compound structure	IC_{50} (nM)[b]	K_d (nM)[c]
1		5000	2000
2		1500	540

[a] HIV-1 RT polymerase and DNA binding inhibition data for two compounds identified by structure-based design.

[b] IC_{50} of HIV-1 RT polymerase as measured in Amersham assay. Errors from plots of time vs. counts per minute for IC_{50} showed variations of 2–10%. Minimization of error was accomplished by comparing timed results of the most significant trials with standards, rerunning with fresh reagents, and applying variation in incubation times. Error variation in scintillation counting was a maximum of ±0.4%.

[c] K_d of HIV-1 RT–DNA binding inhibition.

calibrated by close proximity spotting. NMR spectra (^{1}H, and ^{13}C) were taken on a GE 300 MHz instrument. Centrifugation was performed at top speed on an International Clinical Centrifuge. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 MHz spectrometer. Chemical shifts were expressed in ppm relative to the used solvent; multiplicity was indicated as s (singlet), d (doublet), t (triplet), and m (multiplet). Electrospray mass spectra were performed by the Protein and Carbohydrate Structure Facility of the University of Michigan, P.C. Andrews, Director.

Chemistry. A general method for diazotization reaction was used for the production of the bis(diazo) inhibitors **3–5** according to a previously reported procedure [28], but with slight modifications to allow the production of salt-free dyes. In particular, Li_2CO_3 and HCl were used instead of Na_2CO_3 and H_2SO_4, respectively, to allow the formation of LiCl salts soluble in ethanol and which could be easily discarded from the washing steps in the course of the work-up. The described procedure scaled from 500 mg aniline afforded dyes, judged pure from the NMR spectra, elemental analysis, and HPLC profiles.

General procedure for the preparation of the dyes **3**, **4**, *and* **5**. A sample of the aryl amines, compound **3**, 4-amino-benzoic acid (500 mg, 3.6 mmol, 1 eq); compound **4**, 4-aminocinnamic acid hydrochloride salt (500 mg, 2.5 mmol, 1 eq); compound **5**, 4-amino-3-methyl benzoic acid (500 mg, 3.3 mmol) was slurried in 2 ml water. Also 2.4 eq of concentated HCl (265 μl for **3**, 185 μl for **4**, and 245 μl for **5**) was added, resulting in the formation of a precipitate. Then, an aqueous solution of 2.5 M $NaNO_2$ (1 eq, 1.44 ml for **3**, 1 ml for **4**, and 1.32 ml for **5**) was slowly dropped at room temperature, allowing the dissolution of the precipitate in the following minutes after the addition. The reaction mixture was then cooled in an ice bath. Then 0.5 eq of carbonyl J (987 mg, 1.8 mmol, for **3**; 685 mg, 1.25 mmol for **4**; and 905 mg, 1.65 mmol for **5**) fully dissolved in a solution of Li_2CO_3 (22 ml of 200 mM Li_2CO_3 for **3**, 35 ml of 96 mM Li_2CO_3 for **4**, and 20 ml of 200 mM Li_2CO_3 for **5**) was added, resulting in a deeply colored solution. The pH of the reaction mixture was adjusted to 9.0 by adding a solution of 96 mM Li_2CO_3 and the reaction mixture was allowed to stir at room temperature for 3 h for **4** and 4 h for **3** and **5** after which it was acidified with concentrated HCl to a pH of approximately 0–1 (1.2 ml for **3**, 0.6 ml for **4**, and 1.2 ml for **5**). The reaction mixture was diluted by addition of water to a total volume of 50 ml and the resulting precipitate was isolated by centrifugation at 4 °C at 6000 rpm using 50 ml disposable centrifuge cones, washed with water (6 cycles of 20 min) and then ethanol when the dye started to dissolve in water (at least 10 cycles of 20 min). The orange-red dyes were dried for at least 24 h under vacuum at reflux of acetone in the presence of $CaCl_2$ as desiccant, yielding the desired compounds as metallic dark colored flakes (with 27% yield for **3**, 35% yield for **4**, and 18% yield for **5**).

Analytical data for compound **3**. ^{1}H NMR (500 MHz, d_6-DMSO) δ 15.82 (s, 2H), 12.92 (broad s, 1.5 H, exchangeable with H_2O), 9.53 (s, 2H), 8.22 (d, $J = 8.0$ Hz, 2H), 8.00 (d, $J = 8.0$ Hz, 4H), 7.83 (d, $J = 8.0$ Hz, 4H), 7.76 (d, $J = 8.0$ Hz, 4H), 7.51 (s, 2H), 3.48–3.40 (m, H_2O, C_2H_5OH), 1.07 (t, C_2H_5OH). ^{13}C NMR (125 MHz, d_6-DMSO) δ 178.53, 167.55, 152.51, 146.87, 145.36, 144.83, 137.86, 131.46, 130.15, 129.31, 127.48, 125.65, 122.50, 118.38, 117.26, 116.97, 56.69 (C_2H_5OH), 19.23 (C_2H_5OH). Negative

ESMS: found 799.6 (MH^-). Anal. Calcd. for $C_{35}H_{22}O_{13}N_6S_2Li_2$, $2H_2O$, $2C_2H_5OH$: C, 49.79; H, 4.07; N 8.93. Found: C, 50.60; H, 3.58; N, 8.99.

Analytical data for compound **4**. ^{1}H NMR (500 MHz, d_6-DMSO) δ 15.95 (s, 2H), 12.10 (broad s, exchangeable with H_2O), 9.51 (s, 2H), 8.20 (d, $J = 8.6$ Hz, 2H), 7.77 (m, 12H), 7.61 (d, $J = 15.9$ Hz, 2H), 7,49 (s, 2H), 6.54 (d, $J = 15.9$ Hz, 2H), 3.47 (m, H_2O, C_2H_5OH), 1.07 (t, C_2H_5OH). ^{13}C NMR (125 MHz, d_6-DMSO) δ 177.89, 168.59, 152.75, 145.29, 144.96, 144.82, 144.33, 137.90, 132.18, 130.54, 129.87, 129.30, 125.87, 122.15, 118.96, 118.64, 118.27, 117.092. Negative ESMS: found 851.7 (MH^-). Anal. Calcd. for $C_{39}H_{26}O_{13}N_6S_2Li_2$, $2H_2O$, $2C_2H_5OH$: C, 52.02; H, 4.26; N, 8.46. Found: C, 54.41; H, 3.56; N, 7.81.

Analytical data for compound **5**. ^{1}H NMR (500 MHz, d_6-DMSO) δ 16.22 (s, 2H), 12.83 (broad s, 2H, exchangeable with H_2O), 9.57 (s, 2H), 8.26 (d, $J = 8.0$ Hz, 2H), 8.11 (d, $J = 8.0$ Hz, 2H), 7.91 (s, 2H), 7.89 (d, $J = 9.0$ Hz, 2H), 7.79 (s, 2H), 7.76 (d, $J = 8.0$ Hz, 2H), 7.55 (s, 2H), 3.52–3.45 (m, H_2O, C_2H_5OH), 2.51 (s, 6H), 1.07 (t, C_2H_5OH). ^{13}C NMR (125 MHz, d_6-DMSO) δ 178.67, 167.86, 152.70, 145.58, 144.99, 144.83, 138.00, 132.92, 131.09, 129.61, 129.38, 127.48, 125.96, 125.78, 122.79, 118.62, 117.13, 116.21, 56.88 (C_2H_5OH), 17.53, 19.43 (C_2H_5OH). Negative ESMS: found 827.5 (MH^-). Anal. Calcd. for $C_{37}H_{26}O_{13}N_6S_2Li_2$, 2 H_2O, 1.5 C_2H_5OH: C, 50.80; H, 4.16; N, 8.89. Found: C, 50.61; H, 3.67; N, 8.61.

Amersham polymerase assay. Enzyme activity was measured with a scintillation proximity assay [32], which uses a biotin/streptavidin bead capture system. RNA-dependent DNA polymerase activity was measured using purified HIV-1 RT (Worthington Biochemical, Lakewood, NJ) and a synthetic 17 mer/50 mer RNA–DNA template–primer containing biotin at the 5′ end of the DNA. For initial screening, all inhibitors were dissolved in <10% DMSO. The template–primer, buffer, dNTP, and inhibitor were incubated for 10 min at 37 °C before the RT was added. In initial screening assays, the final 100 μl reaction mixture contained: 39 nM of template–primer; buffer of 40 mM Tris–HCl, pH 8.0/10 mM $MgCl_2$/60 mM KCl/10 mM dithiothreitol; 75 μM of each dNTP except dTTP; 25 μM dTTP and 35 μM tritium labeled d*TTP; 10 μM inhibitor; and 2.0 μg RT. After 3 min of incubation at 37 °C, the reaction was quenched with 40 μl of 0.56 M EDTA. Then, 10 μl streptavidin SPA beads (in suspension) was added and incubated at 37 °C for 10 min. Finally, 850 μl of 10 mM Tris–Cl, pH 7.4, 0.15 M NaCl was added. The signal was produced by biotinylated polymers that contained d*TTP binding to the streptavidin beads and stimulating the scintillant. The amount of d*TTP polymerized was measured using a scintillation counter. Results for each concentration of inhibitor were recorded as counts per minute relative to an uninhibited standard.

NCI polymerase assay. The assay has been previously described [35]. Briefly, RNA-dependent DNA polymerase activity was assayed using purified RT protein and a poly(rC)·oligo(dG), 0.02 mM dGTP, 0.002 mCi [α-^{32}P]dGTP, and a buffer of 25 mM Tris–Cl, pH 8.0, 75 mM KCl/8.0 mM $MgCl_2$/2.0 mM dithiothreitol/10 mM 3-[(3-cholamidopropyl)dimethylammonio-1-propane-sulfonate (CHAPS) containing acetylated bovine serum albumin at 100 μg/ml. The assay mixture was incubated for 30 min and then the reaction was stopped by the addition of 50 μL of a 10-mg/ml solution of sheared and denatured salmon sperm DNA, followed by 3.0 ml of 10%

trichloroacetic acid (TCA). The labeled polymer was collected by suction filtration on Watchman glass GF/C and counted.

Resistant mutants. The mutants were constructed using *Bsp*MI cassette mutagenesis, as described previously [33,34]. The RNA-dependent DNA polymerase activity was measured with the method described above for the NCI polymerase assay.

RT–DNA binding. The oligonucleotides used for attachment to the surface plasmon resonance surface (SPR) were synthesized with biotin at their 3′ ends using TEG CPG (Glen Research, Sterling, VA). The biotinylated plus strand oligonucleotide (5′-A GCA GTG GCG CCC GAA CAG GGA CCT GAA AGC-3′ biotin) was mixed with equimolar minus strand (3′-GGGCTTGTCCCTGGACTTTCG-5′), heated to 95 °C, and allowed to cool to room temperature for annealing. This forms a DNA version of a primer/template that contains sequences from the LTR of HIV-1.

SPR was performed with a BIAcore instrument manufactured by Biosensor AB (Uppsala, Sweden) using methods described previously [36]. The buffer used in the SPR experiments was 0.15 M NaCl–10 mM Hepes (pH 7.5), 5 mM DTT–0.05% Tween 20. Binding experiments were initiated by passing buffer across an SPR sensor chip containing a known amount of oligonucleotide for approximately 100 s at 5 μl/min, followed by a 10 μl injection of buffer containing RT solution. Injection of the RT sample was followed by buffer for an additional 200 s. The chip surface was then regenerated with two successive 5 μl pulses of 0.1% sodium dodecyl sulfate (SDS)–3 mM EDTA.

A standard curve relating the initial binding slope [37] of RT binding to 225 resonance units of primer–template was constructed by injecting solutions containing 0.5. 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 nM HIV-1 RT. Solutions of 500 nM HIV-1 RT were incubated with different concentrations of inhibitor and free HIV-1 RT was measured by injection of the solution and determination of the resultant initial slope. The free HIV-1 RT was plotted against inhibitor concentration and the curve was fitted using the solution affinity model contained in the BIA software version 3.01 to obtain K_d's.

RNaseH assay. The assay for RNaseH activity has been described elsewhere [38,39].

Specificity assay. T4 DNA polymerase, T7 DNA polymerase, the Klenow fragment of *Escherichia coli* DNA polymerase I, and MLV RT were all purchased from New England Biolabs and assayed in the buffers supplied by the manufacturer. HIV-1 RT was assayed in conditions described above for the NCI polymerase assay. Assays were performed for 30 min in the presence of the indicated amount of the inhibitors. The reactions were topped by the addition of 50 μL of sheared salmon sperm DNA and 3.0 ml of 10% TCA. The labeled DNA was collected by suction filtration on Whatman GF/C glass filter and counted in a liquid scintillation counter.

Cell culture. The viral replication assay has been described elsewhere [40]. This assay generates both an effective concentration for cell rescue from viral infection (EC_{50}) and a toxic concentration for cell death independent of viral infection (TC_{50}). From these EC_{50}s and TC_{50}s, a therapeutic index (TI) was calculated (TC_{50}/EC_{50}).

3. Results

We examined three binding sites on the crystallographic structure of HIV-1 RT with the structure-based design program DOCK. We used DOCK to filter a database of compounds from the Available Chemical Directory (ACD) and identify candidate ligands, which were complementary to one of the sites. These three sites are located (i) adjacent to the polymerase active site, (ii) at the base of the thumb, and (iii) in the area between the thumb and the minor groove of DNA. Ninety-two compounds were selected for the three binding sites and screened for their ability to inhibit HIV-1 RT polymerase activity. Twenty-seven of the ninety-two compounds (29%) showed at least 5% inhibition in a 10 μM assay and corresponding increases in inhibition at 30 and 100 μM. The most potent compound from our initial screening was **1** with an IC_{50} of 5 μM (Table 1), while 9-chloro-thiobenzimidazolone (9-Cl-TIBO) in the same assay conditions had an IC_{50} of 20 μM. This overall approach is outlined in Fig. 1.

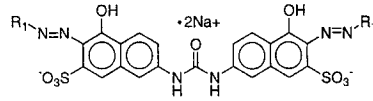
To demonstrate that **1** does not bind to the nucleoside or the non-nucleoside binding site, we assayed it against a variety of HIV-1 RT drug resistant mutants including seven NNRTI resistant single mutants (Y181L, Y188L, L100I, K103N, V106A, E138K, and P236L), two NRTI resistant mutants (L74V and M184V), and the AZT-21 mutant (M41L, D67N, K70R, T215Y, and K219Q). Compound **1** was as effective against all 10 resistant mutants tested as it was against wild-type HIV-1 RT (data not shown).

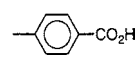
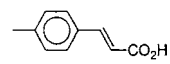
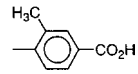
Based on these results, we pursued similar available compounds and identified compound **2**, a superstructure of compound **1**. Compound **2** had an IC_{50} of 1.5 μM versus HIV-1 RT polymerase activity (Table 1). In addition, the new structural motifs found within compound **2** inspired the synthesis of a wide range of aryl diazo derivatives including compounds **3–5**. We also replaced the central urea with a variety of linkers including thiourea, oxalyl, squarate, chelidonate, chelidamate, 2,6-pyridine dicarboxylate, and terephthalate groups. Finally, we modified the linkers and aryl diazo groups together in a combinatorial fashion. The most potent compounds contained urea linkers and carboxylic acid groups (**3–5**, Table 2). This potent inhibition of HIV-1 RT polymerase activity was verified by two independent methods. The Amersham assay and the NCI assay agreed to within a factor of 4 for each molecule. Compound **4** was the most potent compound with IC_{50}s of 90 and 24 nM, respectively, in the two assays (Table 2).

We explored the ability of each of the compounds to inhibit binding of HIV-1 RT to DNA using a BIAcore assay. A prerequisite for this study is that the compounds do not bind to the CM5 chip. Unfortunately, **5** bound to the surface of the chip so its K_d could not be determined. We were, however, able to measure dissociation constants for **1**, **2**, **3**, **4**, and 8-Cl-TIBO. 8-Cl-TIBO, the negative control, did not inhibit RT binding to DNA at any concentration measured. Our initial compounds, **1** and **2**, inhibited RT binding to DNA with K_d values of 2 and 0.54 μM, respectively (Table 1). The more potent compounds, **3** and **4**, inhibited RT binding to DNA with K_d values of 105 and 40 nM, respectively (Table 2).

Compounds **4**, **5**, and 8-Cl-TIBO were tested for their ability to inhibit HIV-1 RT RNaseH activity. Each compound was tested at concentrations of 25, l00, 500, and

Table 2
HIV-1 RT in vitro inhibition[a]



No.	R_2	HIV-1 RT polymerase IC_{50} (nM)[b]	HIV-l RT polymerase IC_{50} (nM)[c]	RNaseH IC_{50} (nM)	DNA binding K_d (nM)
3		390	316	ND[d]	105
4		90	24	25–100	40
5		150	42	25–100	INC[e]

[a] In vitro inhibition of HIV-1 RT polymerase activity (two independent assays), inhibition of HIV-1 RT RNaseH activity, and inhibition of DNA binding to HIV-1 RT.
[b] Data obtained from the Amersham HIV-1 RT polymerase assay. For discussion of error estimates, see Table 1, footnote b.
[c] Data obtained from the National Cancer Institute HIV-1 RT polymerase assay.
[d] Not determined.
[e] Compound **5** was incompatible with the assay procedure.

2000 nM at a 4 min time interval (Fig. 2). 8-Cl-TIBO showed no RNaseH inhibition at concentrations up to 50 mM (data not shown). Compounds **4** and **5** each demonstrated an IC_{50} between 25 and l00 nM (Fig. 2, Table 2), showing that the two most potent polymerase inhibitors are also potent RNaseH inhibitors.

Having identified several potent inhibitors, we investigated their selectivity. Compounds **4** and **5** were tested for inhibition of two viral reverse transcriptases (HIV-1 RT and MLV RT) and three prokaryotic DNA polymerases (T4 DNA polymerase, T7 DNA polymerase, and the Klenow fragment). Compounds **4** and **5** showed significantly more inhibition of the retroviral RTs, HIV-1 RT, and MLV RT, than the T7, T4, or the Klenow DNA polymerases at 200 nM (95% confidence interval, Fig. 3). Indeed, neither of the compounds showed inhibition of T7, T4, or the Klenow DNA polymerase activities at concentrations up to 200 nM. Compounds **4** and **5** are potent and selective inhibitors of retroviral RTs.

Finally, we tested our most promising compounds in a cell culture assay. The EC_{50} values of compounds **3**, **4**, and **5** are 8.8, 2.5, and 2.5 μM, respectively, in an assay of cellular rescue from viral infection (Table 3). The corresponding toxicity measurements for compounds **3**, **4**, and **5** are >100, 112, and 120 μM, respectively, with therapeutic indices of 10–50, indicating that these compounds are not toxic to the cells at concentrations an order of magnitude higher than their EC_{50} values (Table 3).

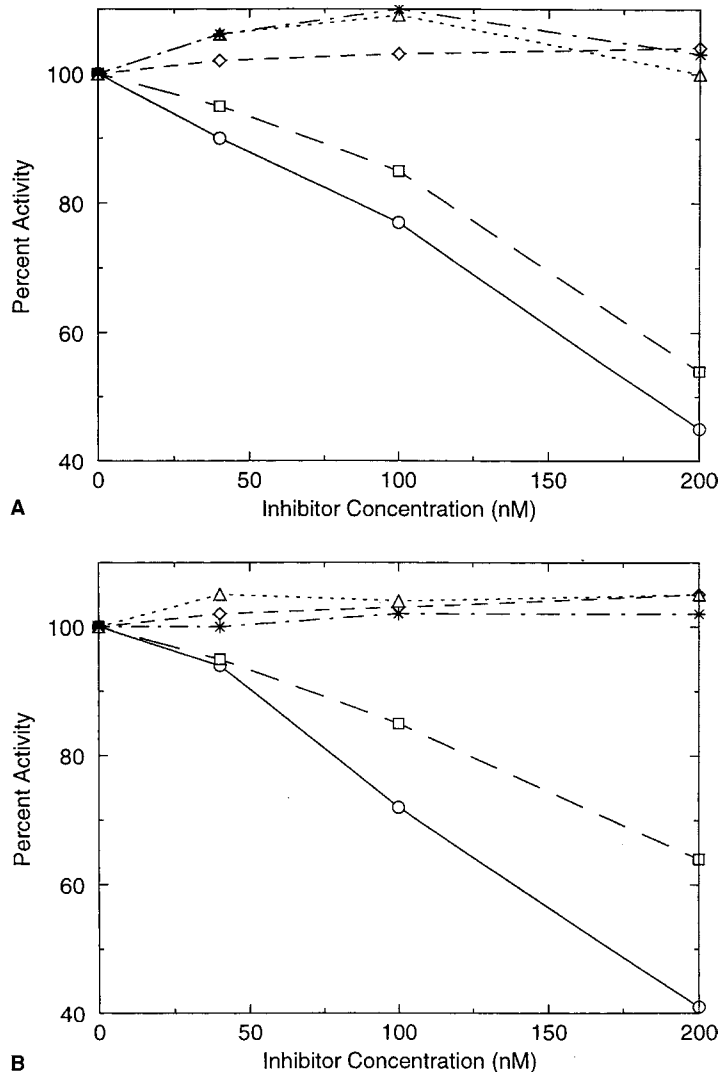
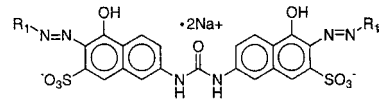


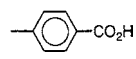
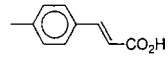
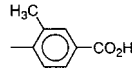
Fig. 3. Selective inhibition of retroviral RTs. Inhibition of HIV-1 RT, MLV RT (-○-), T4 DNA polymerase (-□-), T7 DNA polymerase (-◇-), and Klenow fragment (-△-) by compound **4** (A) and compound **5** (B) (-∗-).

4. Discussion

HIV infection and AIDS remain a major medical problem [2]. The rapid development of resistance to current drug therapies serves to highlight the need for new therapies, which have independent mechanisms of action. We have used structure-based design principles to identify novel, potent, and selective inhibitors of HIV-1 RT. These inhibitors appear to act by a unique mechanism and are not susceptible to

Table 3
HIV-1 cell culture inhibition[a]



No.	R1	EC_{50} (μM)	TC_{50} (μM)	TI[b] (TC_{50}/EC_{50})
3		8.8	>100	>11
4		2.5	112	45
5		2.5	120	48

[a] Effects of compounds **3–5** on cell culture HIV-1 infection (EC_{50}) and cellular toxicity (TC_{50}), and therapeutic index (TC_{50}/EC_{50}) are shown.
[b] Therapeutic index.

the resistance mutations, which eliminate the activity of NNRTIs or NRTIs. Three specific topics merit further discussion. First, we consider the mechanism of action of these naphthylurea derivatives. Second, we examine the utility of structure-based design in a system as challenging as HIV-1 RT. Finally, we discuss the structural and medicinal properties of these compounds.

Mechanism of action. Let us summarize the evidence that these compounds inhibit HIV-1 RT by a novel mechanism. The BIAcore data (Tables 1 and 2) are direct evidence that strongly support the hypothesis that this series of compounds prevents HIV-1 RT from binding to DNA duplexes. In sharp contrast, neither NNRTIs nor NRTIs inhibits such binding. It is well known that RNA–DNA and DNA–DNA duplexes bind HIV-1 RT in a similar manner. Thus, the BIAcore data make the decisive prediction that if these compounds also prevent HIV-1 RT from binding DNA–RNA duplexes, the RNaseH activity of HIV-1 RT should be inhibited by these compounds in a similar concentration range. The RNaseH data (Fig. 2) clearly show that compounds **4** and **5** also inhibit HIV-1 RT RNaseH. Although NNRTIs like Nevirapine can alter the specificity of RNaseH cleavage, they do not block RNaseH activity [41]. Furthermore, in our control, 8-Cl-TIBO had no effect on RNaseH activity (data not shown). Thus, although the binding site and exact binding modes remain unknown, we have shown that these compounds interact with HIV-1 RT and prevent its binding to DNA.

The data presented here imply that these compounds prevent HIV-1 RT's normal interaction with nucleic acid duplexes. The three most plausible mechanisms for this inhibition are (1) binding to RT in a mode competitive with the binding of the nucleic acid duplexes, (2) binding to a secondary site and thereby preventing an essential conformational change required for duplex binding, or (3) binding to DNA and directly interfering with its interaction with HIV-1 RT. The specificity data in Fig. 3 suggest that compounds **4** and **5** are specific RT inhibitors. In addition, these

specificity data reinforce the BIAcore data, indicating that they do not act by binding to DNA. If these compounds inhibited RT by binding to nucleic acids, they would inhibit the three DNA polymerases in addition to the two viral RTs. The enzymatic inhibition, DNA binding inhibition, and selectivity of this class of compounds set them apart from all previously identified HIV-1 RT inhibitors.

Compounds **3**, **4**, and **5** all inhibit HIV-1 replication in cell culture without causing cellular toxicity. Although these compounds are all potent HIV-1 RT inhibitors in vitro, our data do not exclude alternate modes of action in cell-based assays. For instance, unrelated aromatic polysulfonic acids have been shown to inhibit GP-120 binding to CD-4 [42]. However, the compounds presented here are potent inhibitors of HIV-1 RT activity in vitro rather than being identified by high-throughput cellular screening. The EC_{50}s of **3**, **4**, and **5** in culture are one to two orders of magnitude higher than the corresponding in vitro measurements. This is not unusual for small-molecule enzyme inhibitors and may be due to poor cell permeability, cellular efflux pumps, or cellular modification of the compounds. Compounds **4** and **5** may be present in concentrations more than 40 times their EC_{50} without generating cytotoxicity, and thus, are suitable for further cell-culture study.

The compounds with inhibitory properties closest to the series presented here are the marine natural products Toxiusol and Peyssonols A and B [15,17]. Our compounds potently inhibit HIV-1 RT polymerase activity, RNaseH activity, and DNA binding. In contrast, Peyssonols A and B do not inhibit HIV-1 RNaseH activity and Toxiusol has not been reported to inhibit HIV-1 RNaseH activity. Furthermore, our compounds are selective for retroviral RTs, whereas Toxiusol inhibits the Klenow fragment. It is clear that the naphthylureas presented here represent a new direction in RT inhibition.

Structure-based design. HIV-1 RT has presented a difficult crystallographic challenge. However, the original RT–DNA structure [23] contained sufficient information to allow DOCK to identify 27 diverse inhibitors of HIV–RT polymerase activity including **1**, which was sufficiently potent (IC_{50} 5 μM) to merit further development. These results demonstrate that structure-based screening can be useful, even with target structures whose resolution is >2.5 Å.

The NNRTIs encompass a wide array of chemical classes which all bind to the same site and are associated with a characteristic set of resistance mutants [8]. In the design phase, we biased binding site selection away from the non-nucleoside binding site and biased compound selection away from known inhibitors. This biased design strategy successfully identified **1**, which was equally effective against wild-type HIV-1 RT and HIV-1 RT mutants resistant to either NNRTIs or NRTIs. This evidence indicates that **1** does not bind to the non-nucleoside binding site and that structure-based design can be used to specifically target or avoid a particular site on a large macromolecule such as HIV-1 RT.

Although our attempts to grow co-crystals have not yet been successful, the structure of a complex of any of these compounds bound to HIV-1 RT would provide a useful starting place for drug design. Until such data are available, we are exploring several hypothetical binding modes generated by docking the inhibitors to the structure of the HIV-1 RT–DNA complex (personal communication, Jacobo-Molina). One promising binding site is a deep groove at the interface of the p66 and p51

subunits near residues W406, Q507, and A508 of p66, and N418 of p51. However, the binding site and exact binding modes of our compounds remain unknown (except that the compounds do not appear to bind to the NNRT or NNRTI binding sites).

Medicinal chemistry. Compound **2** and its analogs **3–5** are potent superstructures of compound **1**. Although the high degree of symmetry in the best inhibitors is somewhat surprising, the larger compounds presumably are more potent because the additional groups are able to explore interactions in pockets adjacent to the original binding pocket. On the other hand, it is not uncommon for analogs of low potency inhibitors (such as **1**) to explore alternative binding modes. Although we synthesized compounds with a wide variety of central linkers and aryl diazonium side-chains (Fig. 1), the compounds that are most active against HIV-1 RT were the urea-linked compounds with acidic aryl diazo side-chains (**3–5**). All of these compounds are quite potent in vitro. In their current form, however, this class of inhibitors have some drawbacks, including multiple formal charges, diazonium groups, and high molecular weights. When we replace the distal acids with an isoelectric (and medicinally favored) tetrazole, it retains most of its activity (HIV-1 RT polymerase $IC_{50} = 220\,nM$, data not shown). However, we were not able to identify potent smaller or non-sulfated molecules. In order for these compounds to be suitable for study in animal models, further medicinal chemistry development will be necessary. Nevertheless, these compounds are the first of what appears to be a novel class of HIV-1 RT inhibitors.

Aspects of the structure of these compounds appear intriguingly similar to a DNA base pair and one can imagine that they could bind to HIV-1 RT in a similar manner. For example, in a planar conformation, the inter-sulfate distance is within the same range as an inter-phosphate distance across a base pair. The conjecture that this series bind to HIV-1 RT as DNA mimics suggests that they may competitively inhibit nucleic acid binding. Further, if these compounds prove to be examples of a broader class of DNA mimics, new analogs may bind specifically to other DNA binding proteins such as retroviral integrases or host transcription factors.

In conclusion, we have designed and developed a new series of HIV-1 RT inhibitors. These compounds and their mechanism of action are distinct from any previously described class of HIV-1 RT inhibitors. Using a structure-based design strategy biased away from previously known drugs and binding sites, we identified compounds that prevent HIV-1 RT from binding to nucleic acids. This class of compounds are both potent and selective inhibitors of viral reverse transcriptases and are not effected by any of the major HIV-1 RT resistance mutants, which were tested. The data we present demonstrate that HIV-1 RT has a previously undescribed binding site, which is capable of supporting potent small molecule binding and inhibition. The naphthylurea compounds identified here represent an exciting new direction in HIV-1 RT inhibitors.

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